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STUDIES ON TRANSFERENCE OF BONE

III. Manifestations of Immunological Tolerance to Implants of Homologous Cortical Bone in Rats

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The induction of tolerance to homologous cortical bone grafts presents a special problem because bone as a grafting material differs in a significant way from soft tissues. The failure of soft tissue homografts is brought about by an inflammatory process and cellular invasion from the host with subsequent death of the cellular elements of the graft (Medawar, 1944, 1958). The importance of the cellular component of this inflammatory reaction in the destruction of homologous cells has been demonstrated by Weaver, Algire and Prehn (1955). The osteocytes in cortical bone however occupy a position which protects them against this reaction and they survive provided their nutrition is unimpaired (Chalmers, 1959 ; Heslop, Zeiss and Nisbet, 1959*a*). Nevertheless, there are other features by which a homograft of cortical bone differs widely from an autograft, and which render it " unsuccessful ". These features are delayed vascularization, inferior vascularization at all stages, altered development of the granulation tissue formed in relation to the graft, and impairment both of new bone formation, and resorption and replacement of the graft (Heslop, Zeiss and Nisbet, 1960 ; Zeiss, Nisbet and Heslop, 1960).

It has been shown that the reactivity against homogenous soft tissue grafts, such as skin (Billingham, Brent and Medawar, 1956), adrenal (Medawar and Russell, 1958), thyroid and adrenal (Woodruff and Sparrow, 1958) pituitary and ovary (Martinez, Smith and Good, 1958) is weakened or suppressed in animals immunologically tolerant to the donor. Such grafts largely survive and function. The evidence from our earlier work suggests that suppression of the inflammatory reaction alone would be insufficient to make homogenous cortical bone grafts as successful as autografts. The purpose of this experiment was therefore to find out whether induction of tolerance would (1) suppress the inflammatory reaction to homogenous cortical bone grafts, (2) overcome the slowness and inferiority of the vascularization, (3) prevent the abnormal development of the granulation tissue,

and (4) allow unimpaired new bone formation and also resorption and replacement of the graft.

MATERIALS AND METHODS

Induction of tolerance.—Wistar rats were injected subcutaneously within 24 hr. of birth with a suspension of spleen cells (Woodruff and Simpson, 1955) from 4-weeks-old hooded rats, 1 donor rat supplying cells for 2 recipients. The animals received 2×10^7 cells which were contained in 0·1 ml. of fluid, each sample of spleen cell suspension being adjusted to this concentration after a cell count had been carried out.

Check of tolerance.—Surviving Wistar rats received pinch skin grafts from their original hooded donors 12 weeks after spleen cell injection. Split skin grafts (1 sq. cm.) were transplanted to a number of animals intended for vascular injection 14 days after bone implantation.

The extent to which the animals had been made tolerant was assessed by weekly clinical examinations of the skin grafts during 2 months. The state of tolerance was judged by the macroscopic appearance of the grafts, *i.e.* by scabbing and hair growth, but more particularly by their vasculature as seen with the dissecting microscope (Taylor and Lehrfeld, 1953). The skin grafts were similarly examined prior to killing the animals. According to the presence or absence of vascular dilatation or complete thrombosis of the graft vessels, the animals were classified as highly tolerant, moderately tolerant or non-tolerant.

Implantation of bone.—Simultaneously with the application of the skin grafts (12 weeks after spleen cell injection) recipients were implanted with 2 tubular cortical bone grafts from their original spleen cell donors. Operation techniques and graft preparation were those previously described (Heslop *et al.*, 1960). Those rats intended for vascular injection 14 days after bone transfer received 2 split cortical bone implants (derived from one tibia) 8 weeks after skin grafting. At this time the healthy condition of their skin grafts indicated a highly tolerant state.

Excision of bone implants.—Two biopsies were carried out in those animals bearing 2 tubular bone implants, one of the implants being excised within 14 days after implantation, the remaining one at a later date, in order to allow each animal to survive long enough for a retrospective estimate of its tolerance at the time of the first biopsy.

Tubular implants were excised 2, 3, 4, 7, 10, 12, 14, 35, 63, 100 and 250 days after transfer for histology. The animals with split tibial implants for vascular study were killed and injected intra-arterially with Berlin blue at 14 days. The method of injection has been described previously (Zeiss *et al.*, 1960).

Numbers of specimens.—Tubular grafts (8–18) were obtained on each day for histology. However as tolerance was achieved only in a certain percentage of animals, the number was reduced to 4–10 suitable specimens per day. Eventually a total of 69 bone implants from rats

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EXPLANATION OF PLATES

FIG. 1.—Homograft 14 days (highly tolerant host). Copious osteogenesis in highly vascular granulation tissue. The tissue is haemorrhagic but contains no inflammatory cells. ×49.

FIG. 2.—Homograft 63 days (highly tolerant host). Copious osteogenesis in medullary cavity. Remodelling is retarded, and the quantity of bone is in excess of that found in the equivalent autograft. Some haemopoietic marrow remains. ×52.

FIG. 3.—Autograft 63 days. Remodelling of the bone in the medullary cavity has brought about a considerable reduction in its quantity. The marrow is largely fatty. Compare with Fig. 2. ×21.

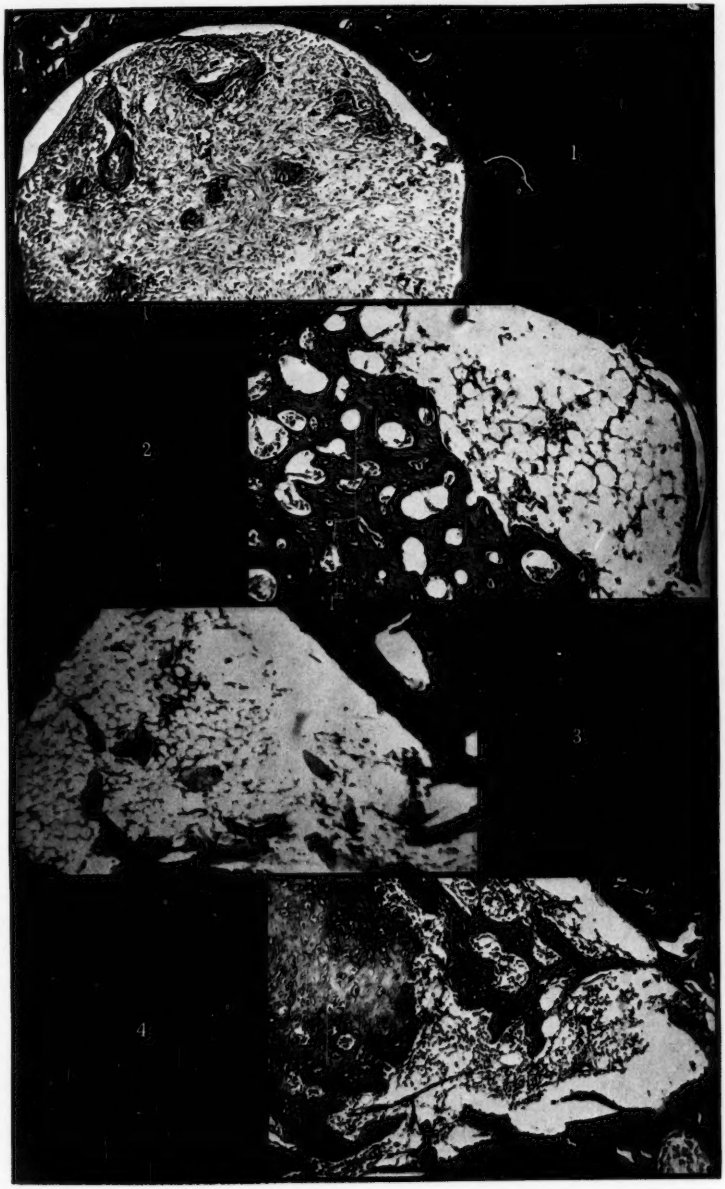
FIG. 4.—Homograft 35 days (highly tolerant host). Extensive development of cartilage is seen along with new bone and haemopoietic marrow. ×49.

FIG. 5.—Homograft 63 days (highly tolerant host). Extensive osteogenesis with retardation of remodelling. The island of collagenous tissue is reminiscent of that associated with homografts. ×48.

FIG. 6.—Homograft 14 days (moderately tolerant host). Osteogenesis associated with a mild inflammatory reaction. ×53.

FIG. 7.—Homograft 14 days (highly tolerant host). The graft is fully vascularized. Vessels injected with Berlin blue and Spalteholz cleared. ×41.

FIG. 8.—Homograft 14 days (non-tolerant host). Poorly vascularized (compare with Fig. 7). Vessels injected with Berlin blue and Spalteholz cleared. ×41.



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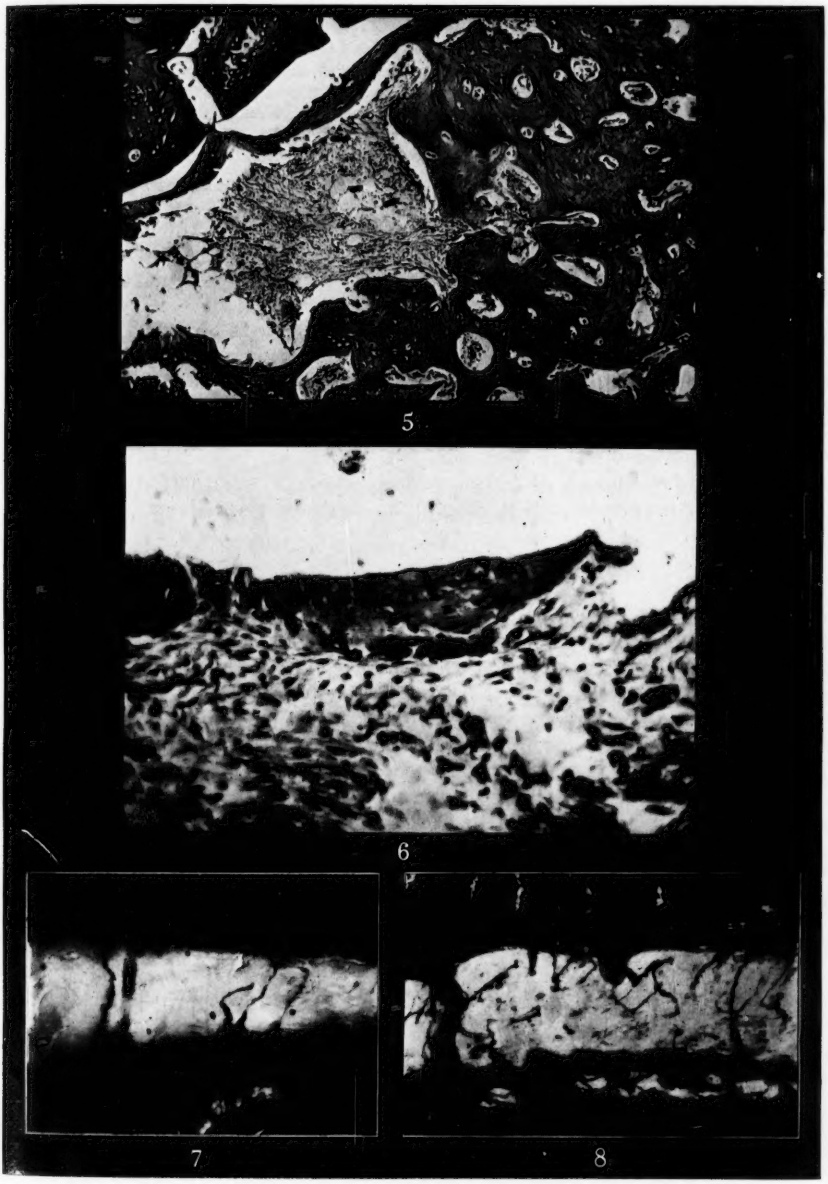

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known to be tolerant was available. For vascular study 10 split implants, representing 5 donor-host combinations, were obtained from apparently tolerant animals.

Assessment of results and controls.—The various items under investigation, *i.e.* the homograft reaction, survival of osteocytes, osteogenesis and vascularization were assessed by methods identical with those used in our previous experiments on autografts and homografts, which also served as controls (Heslop *et al.*, 1960 ; Zeiss *et al.*, 1960).

RESULTS

Effectiveness of spleen cell injection

Twelve weeks after injection of spleen cells 128 rats survived and received test skin grafts. Six of the grafts were technical failures and were lost shortly after application. Of the remaining 122 skin grafts 33 (27 per cent) were rejected within 14 days, indicating that the immunological reactivity of their recipients was unaffected by the spleen cell injection shortly after birth. A further 8 grafts (7 per cent) were rejected within 3–5 weeks after transplantation. Eight weeks after grafting 35·5 per cent of the animals had rejected their grafts, 36·5 per cent were regarded as being highly tolerant, and the remaining 28 per cent were considered to be moderately tolerant. Of the 46 rats which were allowed to survive for more than 190 days, 42·5 per cent appeared to be highly tolerant and 11·5 per cent were moderately tolerant, as judged by clinical examination of the skin grafts.

Mortality after spleen cell injection

In 195 Wistar recipients injected with spleen cells shortly after birth there were numerous casualties from " runt disease " (Billingham, 1958) and 5 rats died within 3 days of injection. The overall mortality was eventually 21 per cent, having reached 14·5 per cent by 5 weeks and 19·5 per cent by 10 weeks. Thus of all the rats which died, 69 per cent were dead by 5 weeks and 93 per cent by 10 weeks. In 270 untreated Wistar baby rats, 11 (4·1 per cent) died within the first 4 weeks of life and no further mortality occurred. Of the 34 treated rats which died during the early stages of the experiment, 11 noticeably suffered from " runt disease ". Some of them were killed if they were moribund and post mortem examinations were carried out. The following clinical abnormalities were observed : the majority of animals were pale and looked anaemic, but on the contrary some had red ears, paws and tails. In most the fur was thin and rough and the skin showed noticeably reduced elasticity. In extreme cases of the pale disease, the back was strongly arched. The weights of the sick animals were usually much lower than those of their healthy litter mates. In pale rats weight differences of up to 50 per cent were observed. Exudation from the nose was common and animals found snuffling noisily and frequently rubbing their noses soon proved to be severely affected by the disease. Many had diarrhoea and occasionally transient paralysis of the hind legs was observed. From the post mortem findings it was possible to divide the sick rats into 2 groups. The features of the first group, which corresponded to the red variety, included gross enlargement of the spleen and lymph nodes, the spleen often being nodular and of an abnormally dark brown colour. Sometimes the adrenals appeared enlarged. The blood was thick and dark in colour. The marrow was also dark and of gelatinous consistency. Typical appearances of the second group, which corresponded to the pale variety, included atrophy of varying degree of the thymus and lymph nodes, the latter

appearing brown. The spleen usually appeared normal in size. Blood and bone marrow were watery and pale. Many other organs were routinely examined but will not be reported upon here.

Histology of bone implants in highly tolerant animals

On account of the unexpectedly high percentage of animals which did not become highly tolerant, insufficient specimens were available for statistical comparison of this series with the autologous and homologous controls.

In all respects the appearances of the bone grafts closely approximated to those seen in the corresponding autografts of our previous series (Heslop *et al.*, 1960). The average proportion of osteocytes surviving in the implants in highly tolerant animals was invariably higher than that seen in homografts, and on some days even slightly exceeded the percentage surviving in autografts. Osteogenesis similarly followed the pattern seen in autografts (Fig. 1), although the average quantity of bone on a given day (at least during the first 5 weeks) was always somewhat less than that seen in the corresponding autograft. During the later stages remodelling was likewise slower in the tolerant series than in autografts (Fig. 2, 3). For example, a 250 day bone graft in a tolerant animal had usually been remodelled only to the extent seen in 63 or 100 day autografts. Similarly the conversion of haemopoietic to fatty marrow was slower in this group than in autografts. Slightly more tendency to cartilage formation was seen in the tolerant series than in the autologous implants (Fig. 4). Very occasional small numbers of inflammatory cells were seen round the bone implants in highly tolerant animals. These aggregations were, however, negligible by comparison with the extent of the cellular infiltration seen in moderately tolerant animals. The connective tissue was generally morphologically normal, but occasional small islands of collagen were reminiscent of the tissue seen around homografts (Fig. 5).

In spite of the apparent induction of a high degree of tolerance as judged by skin grafting it was exceptional to find a bone graft which was absolutely indistinguishable in all respects from an autograft. Most of the bone implants were either retarded in the extent of their remodelling, or in the case of the earlier specimens, showed minor inflammatory cell infiltration slightly in excess of that seen round autografts.

Histology of bone implants in moderately tolerant animals

The bone implants tended towards homograft behaviour rather than the autograft behaviour seen in the highly tolerant group. The average percentage of surviving osteocytes was lower than that seen in the corresponding highly tolerant animals, but was a little higher than that seen in the equivalent homografts. The average extent of osteogenesis was in excess of that seen in the equivalent homograft, particularly in the later specimens. Remodelling, however, was distinctly retarded, even by comparison with the highly tolerant animals. Haemopoietic marrow persisted considerably longer than in the highly tolerant animals. Many of the moderately tolerant animals showed some osteogenesis but also contained islands of abnormal connective tissue showing the appearances previously described as being characteristic of homografts (Heslop *et al.*, 1960). Where such islands of abnormal connective tissue were present, no osteogenesis was observed within them. The adjacent new bone, however, presented no morpho-

logical abnormality and it was not possible to determine the nature of the tissue which had preceded its appearance. Inflammatory cell infiltration was not infrequent in the moderately tolerant animals, the component cells of these aggregations being identical with those seen round homografts. On the whole the inflammatory cell infiltration tended to be rather sparse by comparison with homografts, although this was not invariably so.

While there was a general tendency for the moderately tolerant animals to treat their bone implants like homografts, *i.e.* by showing diminished osteocyte survival, diminished osteogenesis and a homograft reaction, these features were not invariably present to the same degree. For example, occasional cases showed osteogenesis considerably in excess of that seen round homografts in spite of the presence of a moderate homograft reaction (Fig. 6). Generally, however, there was a close parallelism between the various features indicative of homograft behaviour.

Correlation between skin and bone tolerance

Skin tolerance was assessed clinically in the manner already described. The extent of bone tolerance on the other hand was determined independently by histological means, the state of the skin graft not being known at the time. It was found that in nearly every case a close correlation existed between the histological estimate of bone tolerance and the clinical assessment of skin tolerance. In fact it was sometimes possible to predict the ultimate appearance of the skin graft from the behaviour of an early bone graft.

Vascularization of bone implants

Vessel counts carried out in split bone implants 14 days after insertion showed that the vascularization of homologous bone implants in highly tolerant rats was similar to that in autografts because there was no significant difference in the number of vessels in the various classes in the 2 groups. The number of Class III vessels was even slightly higher in the tolerant group than in the autograft group. The vascularity of the homogenous grafts in highly tolerant animals was much superior to that of homogenous grafts in non-tolerant animals (Fig. 7, 8). The number of vessels in the 2 groups differed significantly in the Classes II, III, IV ($P = <0{\cdot}001$) but not in Class I (see Table).

TABLE.—*Vascularization of Homografts in Tolerant Rats as Compared with that of Autografts and Homografts in Untreated Rats* 14 *Days After Implantation*

		Class of vessels			
		I	II	III	IV
Mean number of vessels in	1. Autografts	23·66	19·05	23·89	4·93
	2. Homografts	24·89	5·78	4·75	1·08
	3. Homografts in tolerant rats	21·02	21·02	38·34	4·50
Diff. mean auto.–tol.		2·64	1·97	12·34	0·43
P		0·4	0·4	0·01–0·02	0·5–0·6
Diff. mean homo.–tol.		3·87	12·24	33·59	3·42
P		0·05–0·1	< 0·001	< 0·001	< 0·001

Class I vessels = Just entering bone.
Class II vessels = Well into bone but not beyond the outer lamellar zone.
Class III vessels = Reaching Haversian zone.
Class IV vessels = Full penetration of cortex.

A comparison between the ratios of short vessels to long vessels in the tolerant group at 14 days and normal bone revealed a significantly higher ratio in the former, suggesting that vascularization was still incomplete but progressing at a high rate.

DISCUSSION

The variety of effects produced by the standard injection of homologous spleen cells in this experiment calls for some explanation. On the one hand 27 per cent of the treated animals showed no sign of tolerance, while on the other hand 21 per cent died, some obviously suffering from runt disease. Some of the remainder were seemingly healthy but highly tolerant and others were healthy but moderately tolerant. Woodruff and Sparrow (1957) using the same strain combination and comparable doses, but an intra-peritoneal injection, obtained a higher mortality (41 per cent) but at the same time a considerably higher incidence of " virtually complete " tolerance. This would suggest that by the subcutaneous route used in this experiment the injected cells reached the sites where they are to take effect too late, thus accounting for those cases in which no tolerance was observed (Billingham, 1958).

Of the 34 spleen cell treated rats which died (21 per cent), only 11 showed obvious signs of " runt disease ". It would, however, seem that in the majority of cases death was in some way caused by the injection of the foreign cells, as only 4 per cent mortality occurred in untreated animals of the same strain reared under the same conditions. Detailed observations relating to the disease will not be discussed here at length, but the opportunity is taken to point out that the findings in our pale rats tally with those described by Billingham and Brent (1957) and Billingham (1958) in mice, and with the anaemia described by Simonsen (1957*a*) in chickens. In our animals splenomegaly and enlargement of the lymph nodes occurred mainly in red rats. If these rats were allowed to survive they later became anaemic with signs of involution of the lymphoid organs, suggesting that the red was an earlier phase of the disease which terminated with severe anaemia. It seems likely that both the syndromes we observed were expressions of a reaction of the graft of adult foreign cells against the tolerant host, as Billingham (1958) and Simonsen (1957*b*) have suggested for " runt disease " and the disease in tolerant chickens respectively. Woodruff and Sparrow (1957) also attributed the high mortality in their spleen cell injected rats to a graft versus host reaction. The striking similarities between our pale and red animals and the pale and red partners in parabiotic union described by Chute and Sommers (1952), Stephenson (1953) and others, seem worth mentioning. In parabionts both red and pale forms of the disease seem to be lethal, as is shown by the conflicting reports in the literature as to which partner dies first.

There seems to be nothing surprising in the finding that homogenous cortical bone grafts in tolerant animals behave like autografts, because tolerance is unlikely to be tissue specific (Billingham *et al.*, 1956) and it has been shown to extend to a variety of soft tissue grafts. There are, however, several important aspects in which the autograft-like behaviour of cortical bone differs from that of soft tissue grafts. The autograft-like behaviour in the latter is explained in part at any rate, by the fact that induction of tolerance suppresses the specific inflammatory reaction, usually designated the homograft reaction, and thus allows the grafts to survive and function.

In the case of cortical bone grafts, this reaction seems to be of less significance, as it has been shown (Chalmers, 1959 : Heslop *et al.*, 1959*a*, 1960) that a considerable number of osteocytes in homogenous bone grafts survive in the presence of such a reaction because of their protected position, and may do so for at least 250 days. In fact the vitality of their component osteocytes is a feature in which autogenous and homogenous cortical bone grafts differ relatively little from each other. Striking differences between the two types of grafts have been observed, however, in connection with (1) the quantity of new bone formed in their vicinity, (2) their vascularization and eventual fate, and (3) the development of the granulation tissue in their concavities (Heslop *et al.*, 1960). From a preliminary study (Heslop *et al.*, 1959*b*) it was concluded that induction of tolerance to bone is possible. However, no detailed report was available concerning its influence on the features mentioned above. The evidence of this experiment now strongly suggests that tolerance does in fact alter them all, and it thus seems to reveal additional aspects of the phenomenon of tissue transplantation tolerance.

In our previous experiments (Heslop *et al.*, 1960) it was shown that the osteogenesis related to the autogenous cortical bone grafts almost certainly arose as a response of host cells to an osteogenic stimulus from the graft. The new bone developed in the granulation tissue which filled the concavities related to the surfaces of the grafts. In the case of homografts, therefore, the following explanation for the absence of osteogenesis was suggested—the granulation tissue in their vicinity, being morphologically abnormal and slow in its growth from the beginning, was an unsuitable precursor material for osteogenesis.

If this explanation were correct, the fact that induction of a high degree of tolerance lead to autograft-like new bone formation, would imply that it abolished or overcame the mechanisms which cause the slow and abnormal development of the granulation tissue around homografts. At 14 days the vascularity of homografts in tolerant rats was also autograft-like, implying that both the destruction of graft vessels in the course of the inflammatory reaction and—what seems more important—the early reluctance of the host to vascularize a homograft were absent. It has been mentioned elsewhere (Zeiss *et al.*, 1960) that the slow and abnormal development of the granulation tissue and the slow vascularization of the homografts, may in fact be manifestations of the same process, which, because of its early onset can hardly be regarded as a consequence of the inflammatory component of the homograft reaction. The present observations that induction of tolerance prevented these vascular and connective tissue abnormalities and thus allowed osteogenesis to occur, would indicate an effect of tolerance which exceeds the mere suppression of the inflammatory reaction to homografts. It would also offer confirmatory evidence for our previous suggestion (Heslop *et al.*, 1959*c*) that the abnormal reparative processes associated with homologous bone are probably no less a part of the homograft reaction than is the inflammatory cell infiltration to which the term more commonly refers. A parallel modification of both features of the host reaction according to the extent of tolerance was seen in the majority of animals, and provides further evidence of a similar aetiological basis for both components.

In the light of this evidence the occasional exceptions to the general pattern of behaviour were particularly puzzling. The fact that an inflammatory homograft reaction was on occasions seen in the presence of fairly extensive osteogenesis was perhaps less surprising than the co-existence of new bone and abnormal connective

tissue. Previous evidence (Heslop *et al.*, 1960) has indicated that the inflammatory cell infiltration *per se* probably exerts no inhibitory effect on osteogenesis. The existence of islands of abnormal connective tissue surrounded by areas of apparently normal ossification was more difficult to account for. It may be that only a proportion of the connective tissue round the implants was abnormal, and hence failed to undergo ossification. On the other hand, all the connective tissue might have been slightly abnormal, allowing limited osteogenesis as compared with autologous controls. The general retardation of osteogenesis and remodelling throughout the whole tolerant series possibly favours the second alternative.

Very little is known at present concerning the mechanisms responsible for the suppression of the inflammatory component of the homograft reaction in tolerant animals (Owen, 1959), and in the light of this, any speculation concerning the modification of the reparative process would be premature.

SUMMARY

Tolerance was induced in Wistar rats by the subcutaneous injection of spleen cells from hooded donors within 24 hr. of birth. When the recipients were adult they were grafted with standard implants of cortical bone from their original donors. The implants and adjacent host tissues were examined histologically at varying times from 2–250 days. The vascularity of split cortical grafts from the tibia was assessed at 14 days. All results were correlated with those of our previous studies on autogenous and homogenous implants in untreated rats.

With the technique employed, a high degree of tolerance was induced in 36·5 per cent and moderate tolerance in 28 per cent of the recipients. " Complete " tolerance was apparently never achieved. In 21 per cent of the animals the neonatal injection of foreign cells resulted in clinical " runt disease " or premature death.

In tolerant animals the reaction of the host to homografts was modified, as a result of which it approximated towards the reaction seen round autografts, the degree of approximation corresponding to the degree of tolerance conferred.

The experiment provides evidence that by induction of tolerance (1) the reluctance of the host to vascularize homogenous cortical bone grafts is overcome ; (2) the inflammatory reaction to the antigenic stimulus of the foreign osteocytes is suppressed ; (3) the granulation tissue is allowed to develop similarly to that round autografts, *i.e.* to be transformed into new bone and marrow ; (4) the host is enabled to replace the foreign cortical bone implant by new bone.

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THE CHEMICAL BASIS OF THE VIRULENCE OF *PASTEURELLA PESTIS*

I. The Isolation and the Aggressive Properties of *Past. pestis* and Its Products from Infected Guinea-pigs

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This paper and those to follow will describe attempts to identify the compounds responsible for the virulence and immunogenicity of *Pasteurella pestis*. Two departures have been made from most previous studies on *Past. pestis*. First, we have fractionated *Past. pestis* isolated directly from guinea-pigs dying of plague. Second, our basis for chemical fractionation has been biological assays for aggressins, toxins and immunogens in guinea-pigs as well as mice. We have not concentrated on apparent serological entities which, either are not connected with virulence or so far have only been shown to be associated with virulence by a study of variants. Some reasons for this approach are as follows.

A puzzling feature of previous studies on the toxicity of *Past. pestis* was the lack of relationship between the susceptibility of guinea-pigs to the infection and to the so-called toxin (Jawetz and Meyer, 1943; Englesberg and Levy, 1954; Ajl, Reedal, Durrum and Warren, 1955; Schar and Meyer, 1956). Although this toxin was lethal for mice it was not so for guinea-pigs (Schar and Meyer, 1956; Schar and Thal, 1955) whereas the two hosts were equally susceptible to plague infection (Jawetz and Meyer, 1943; Pollitzer, 1954). This anomaly suggested that in plague, either guinea-pigs are killed by a toxin different from that killing mice or the "toxin", previously isolated, is a degraded form of a native toxin that kills both species. To remove the anomaly it was essential to obtain a preparation from *Past. pestis* which would kill guinea-pigs, and such material seemed most likely to be found in organisms (and their extracellular products) obtained from guinea-pigs dying of plague.

In vaccination studies on plague, a similar anomalous situation existed which indicated that the currently accepted chemical basis for immunity to plague was as insecure as that for toxicity. It was generally accepted that live attenuated vaccines immunized guinea-pigs as well as mice but dead vaccines (without artificial adjuvant) were relatively ineffective for guinea-pigs although adequate for mice (Girard, 1955; Spivack, Foster, Larson, Chen, Baker and Meyer, 1958). Therefore either the essential immunogen for guinea-pigs was different from that for mice and found only during the growth *in vivo* of living vaccines, or there was a common protective antigen for both species which was so degraded during the preparation of vaccines as to be capable only of protecting mice. There is evidence for these suggestions in previous work on the fractionation of *Past. pestis* for the protective antigen(s). The preparation which has been claimed as the principal antigen for immunizing against plague is Fraction 1 (for brief details of nomenclature see Burrows and Bacon (1958)), a non-toxic component of the

envelope obtained from saline extracts of acetone dried *Past. pestis* by Baker, Somner, Foster, Meyer and Meyer (1952). This preparation immunized mice adequately but it is revealing to compare the immunogenicity in mice of Fraction 1 and other fractions with that of the whole killed organisms (Baker *et al.*, 1952). The initial saline extraction produced a gross loss of activity; the extract and the residue antigen protected mice but neither had the activity per mg. of the original bacteria. Furthermore, the purest Fraction 1 (crystalline Fraction 1B) isolated in 0·8 per cent yield had no more activity per mg. than the original bacteria and if anything had less activity than Fraction 1A isolated in 3·5 per cent yield but containing some carbohydrate. Hence even in mice Fraction 1 as isolated did not account for more than a small proportion of the protective activity of dead *Past. pestis*, and therefore the possibility of Fraction 1 being one of a number of protective antigens or a fragment of the native antigen is at once apparent. In guinea-pigs, Fraction 1, like dead bacteria, had little effect unless injected with an adjuvant. The " residue antigen " had some protective effect for guinea-pigs as well as for mice and appeared to contain a residual amount of the native antigen(s). In view of the shortcomings of the above preparations, organisms grown *in vivo* were investigated for protective antigens which would immunize guinea-pigs without adjuvants and mice, more effectively than isolated Fraction 1.

The aggressive action of *Past. pestis* must at least in part be antiphagocytic since it is generally agreed that in successful protection phagocytosis is seen as a major defence against invasion (Jawetz and Meyer, 1944). Cell free serum from normal and even hyperimmune animals possess little bactericidal action against virulent or avirulent *Past. pestis* (Jawetz and Meyer, 1944). *In vivo*, virulent—but not avirulent *Past. pestis*—rapidly acquire resistance to phagocytosis (Burrows and Bacon, 1954; Fukui, Lawton, Janssen and Surgalla, 1957). The chemical basis for this resistance which *in vivo* is accompanied by capsulation is not clear. Fraction 1 or some complex containing it probably contributes to the phagocytosis-resistance of capsulated organisms since free Fraction 1 has an antiphagocytic action (Chen and Meyer, 1954) but in view of the work of Burrows and Bacon (1956*a*, 1956*b*) compounds containing Fraction 1 do not appear to be the sole antiphagocytic substances. These workers prepared in a simple medium *in vitro*, phagocytosis-resistant *Past. pestis* with no visible capsule from a number of mutants including one apparently incapable of producing Fraction 1 (Burrows, 1957). The resistance—only acquired by virulent strains—was associated with the presence in the culture liquid of two precipitinogens V and W. These components were detected serologically in diffusion plates but their direct responsibility for resistance to phagocytosis has not been proven. The culture filtrate containing them did not inhibit the phagocytic action of PMN's whereas a phagocytosis inhibiting substance was produced *in vivo* (Burrows and Bacon, 1954) and by *in vitro* culture of virulent *Past. pestis* in mouse peritoneal fluid or whole blood (Burrows and Bacon, 1956*a*). Chemical extraction for substances showing antiphagocytic activity of organisms grown *in vivo* and their extracellular products in the host fluids should help to clarify these problems.

This paper describes the collection from infected guinea-pigs of *Past. pestis* in sufficient quantity and of adequate purity for chemical work, also a mild method for the extraction of these organisms, and the aggressive activity of the various bacterial products. The toxicity and immunogenicity of these products will be dealt with in future publications.

MATERIALS AND METHODS

Strains of Past. pestis

Unless otherwise stated, the virulent L37 and the avirulent Tjiwidej (TS) strains were used throughout this work.

Assays for ability to inhibit phagocytosis of Past. pestis

With guinea-pig leucocytes.—The viable count (on tryptic meat agar containing 5 per cent peptic sheep blood) of a suspension of *Past. pestis* (Tjiwidej grown on tryptic meat agar 28°, 18 hr.) was determined before and after its phagocytosis by cells of defibrinated guinea-pig blood in the presence and absence of dilutions of the materials under test. The lowest dilution of these was noted which significantly increased the final viable count over that of control samples. This test, which will detect 2–3-fold differences in activity has been described previously (Keppie, Smith and Harris-Smith, 1953 ; Smith and Gallop, 1956). In the absence of the WBCs neither plasma nor serum from guinea-pigs reduced the viable count in a similar test.

With mouse leucocytes.—Using *Past. pestis* (Tjiwidej) and mouse blood (heparin 10 I.U./ml.) the phagocytic index was determined after rotating a bacteria/PMN mixture (15 : 1) for 1 hr. at 37° under conditions as described by Keppie *et al.* (1953) in work with anthrax. Dilutions of the materials under test, produced a decrease in the phagocytic index compared with control samples and the lowest dilution which produced a significant difference was noted. The assay would detect 2–3-fold differences in activity.

Assay of ability to enhance the virulence of Past. pestis *for guinea-pigs*

A small quantity (0·6 ml. containing 120 viable organisms) of *Past. pestis* (L37 ; grown on tryptic meat agar, 37°, 18 hr.) in gelatin-Locke's diluent was mixed with dilutions (5·4 ml.) of the test material in gelatin-Locke's. After standing for 1 hr. at room temperature the mixture (0·5 ml. containing approx. 10 *Past. pestis*) was injected intradermally into guinea-pigs (300–400 g.). The time between injection and death of the guinea-pigs was noted as also was the lowest dilution of the material producing a significant reduction in the mean survival time of controls. Animals were observed for 8–14 days. We are indebted to our colleague Mr. S. Peto for the statistical analysis.

Serological precipitation in gel-diffusion plates

The technique and system of wells were as described by Burrows and Bacon (1956*b*, 1958) but the gel contained sodium azide (0·25 per cent) as preservative. Unless otherwise stated, the results quoted are those obtained by diffusion at 0–2°. This low temperature was used to ensure as far as possible that no enzymic degradation occurred on the plates. It also produced sharper and more distinct lines than corresponding experiments at room temperature or 37°. Two antisera were used.

Unabsorbed antiserum (L.R.).—Rabbits received intravenously, at approx. weekly intervals the following doses : acetone dried *Past. pestis* (Tjiwidej) 0·25 and 1 mg.; acetone dried L37, *in vivo* grown 1, 5, 10 and 20 mg.; living Tjiwidej 10^9, 10^9, 10^9 and 2×10^9 ; and finally living L37, 10^5, 10^7, 10^8 and 10^8 given i.v. followed by 10^9 and 8 doses of 10^{10} given s.c. Serum was taken from the rabbits 7 days later and was stored at −20° with 0·01 per cent merthiolate as preservative.

Absorbed antiserum (L.R.A.).—For detecting only virulence-antigens, serum L.R. (1 ml.) was absorbed with the avirulent Tjiwidej strain (50 mg. of acetone dried organisms) as described by Burrows and Bacon (1956*a*).

RESULTS

Isolation of Past. pestis *from infected guinea-pigs*

The process already described for isolating *Bacillus anthracis* from infected guinea-pigs (Smith, Keppie and Stanley, 1953) was modified to cope with the fact that when the guinea-pigs died of plague, the majority of the available organisms were in the peritoneal and thoracic cavities (rather than in the blood) and were mixed with only a relatively small amount of a highly viscous haemorrhagic

exudate. To remove the majority of the organisms and to dilute the exudate sufficiently to allow of efficient differential centrifugation of the blood cells, the peritoneum and thorax were washed out with a large volume of heparinized saline. Unlike the experiments on anthrax, no attempt was made to collect undiluted exudate or whole blood unless it was specially required.

Infecting and collecting procedure.—Four hundred large guinea-pigs (at least 800 g.) were injected with *Past. pestis*, strain L37 grown at 28° for 32 hr. in xylose-casein hydrolysate (Druett, Robinson, Henderson, Packman and Peacock, 1956) with shaking. Each animal was given $3{\cdot}5 \times 10^8$ organisms both intrapulmonarily and intraperitoneally. Death of almost all the animals occurred in 36–50 hr. Immediately after death, cold heparinized saline (2 units/ml.) was introduced into the thorax (25 ml.) and abdomen (50 ml.) The animals were then placed on shelves in a perspex safety cabinet, fitted with a forced draught air filtration system. After gentle massage, the animals were slit open along the ventral line and turned over to allow the suspension of *Past. pestis* to accumulate on the sloping floor of the cabinet.

Differential centrifugation.—Subsequent operations were done at 0–2° and a similar apparatus to that described by Smith, Keppie and Stanley (1953) was used to remove supernatants from centrifuged deposits and for the transfer of liquids.

The suspension of bacteria (approx. 1×10^9 in exudate diluted with saline) was collected into centrifuge pots (250 ml.) and centrifuged at 500 rpm (60 ***g***) for 30 min. The supernatants were removed to within $\frac{1}{2}$ in. of the blood cell deposit and *Past. pestis* was collected from pooled supernatants by centrifuging for 45 min. at 18,000 rpm in the batch bowl (No. 1600—capacity 1600 ml.) of the Spinco ultracentrifuge (Model L). All the organisms from 400 guinea-pigs were collected in 2 bowls which were used alternately. Samples of the pooled supernatants (saline/exudate 20–25 l. from 400 guinea-pigs) were filtered through Millipore filters and kept at −20° or freeze dried. For special purposes small quantities of exudate undiluted with saline were collected, freed from bacteria and blood cells by centrifugation, and filtered sterile.

The organisms were resuspended in the bowls by rotating them after adding saline (700–800 ml.) and stainless steel ball bearings. Almost all the remaining blood cells were removed from the suspension by centrifuging it (in 4×250 ml. pots) at 500 rpm (60 ***g***) for 30 min. After the supernatant was removed to $\frac{1}{4}$–$\frac{1}{2}$ in. from the blood cell deposit, the latter was washed with saline (700–800 ml.) and again deposited by centrifuging at 500 rpm (60 ***g***) for 30 min. The 2 supernatant suspensions were mixed and centrifuged for 1 hr. at 18,000 rpm in the batch bowl of the Spinco ultracentrifuge. Samples of the supernatant (saline wash, approx. 3 l. from 2 bowls *i.e.* from 400 guinea-pigs) were filtered through Millipore filters and kept at −20° or freeze dried. Sometimes, if the original exudate was much haemolysed, an additional washing of the organisms with saline (1600 ml.) was done to remove more haemoglobin. The organisms were suspended as above (100–150 ml. water for each bowl) and were then ready for extraction with ultrasonic waves. In 5 experiments yields of 1·2, 1·4, 1·6, 1·7 and $1{\cdot}4 \times 10^{13}$ *Past. pestis*, equivalent to approx. 4·8, 5·6, 6·4, 6·8 and 5·6 g. dry weight respectively, were obtained from 400 guinea-pigs. Counts on the suspension prior to final centrifugation showed the contamination with WBCs and RBCs was less than 0·025 per cent.

Extraction of Past. pestis *with ultrasonic waves.*—The slurry of organisms (40–50 ml. 5–7 $\times$ 10^{10} per ml.) collected as described above was treated with the MSE/Mullard ultrasonic disintegrator (20 kc./s. ; 60 watts, probe end ratio 9 : 1). A special all-glass, jacketted container (approx. 60 ml.) was employed (Fig.). The thick slurry was gently stirred with a magnetic stirrer and maintained at $+3°$ by circulating an ethylene glycol/water mixture maintained at $+1°$ through the jacket with a Stuart Turner pump. As a safety precaution a thick rubber pad was sealed with tape to the probe and to the container ; and the whole operation was conducted in a safety cabinet. Microscopical examination showed that under these conditions, practically no *Past. pestis* organisms remained intact after

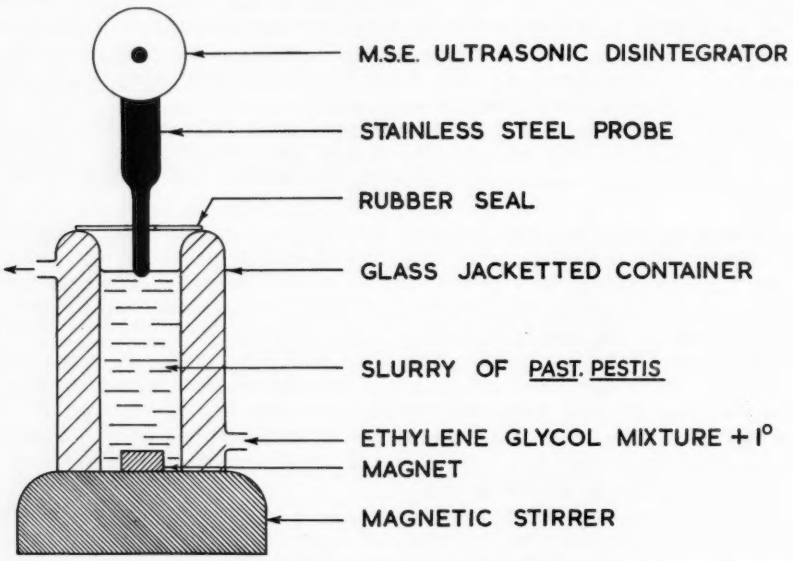

FIG.—A diagram of the apparatus used for extraction of *Past. pestis* with ultrasonic waves.

30 min. After treatment, the slurry was diluted with water to a volume equivalent to 1 $\times$ 10^{10} organisms (approx. 4 mg. dry weight) per ml. and centrifuged at 10,000 rpm for 45 min.

The supernatant—the ultrasonic extract (*US*) constituted approx. 70 per cent by weight of the original organism ; it was filtered through Millipore filters.

The deposit—the ultrasonic residue (*R*) consituted approx. 30 per cent by weight of the original organism ; it was washed twice with water (vol. equal to that of the original diluted slurry) and centrifuged at 10,000 rpm for 45 min. after each washing. At a concentration $\equiv$ 1 $\times$ 10^{10} *Past. pestis* per ml. R was used in 3 forms (*a*) suspended in saline, (*b*) as (*a*) but the few remaining *Past. pestis* were killed by heating the suspension to 60° for 1 hr., (*c*) in solution ; over 95 per cent of R could be dissolved and the remaining *Past. pestis* killed by a short treatment with alkali as will be described in a later paper.

Serological precipitation of the products of Past. pestis *grown* in vivo

The following preparations were examined on serological diffusion plates against unabsorbed and absorbed antisera.

Whole organisms.—These were examined for us by our colleagues M. J. Crumpton and D. A. L. Davies by placing a suspension of live or acetone dried organisms (Crumpton and Davies, 1956) in the antigen wells. Not only did they examine the strain (L37) which has been used throughout this work, but also the virulent MP6, Yokohama and Shasta strains which were grown *in vivo* and isolated in small quantity. The usual spectrum of precipitation lines (Crumpton and Davies, 1956) was obtained against unabsorbed serum. Against absorbed serum all strains showed a total of 4 lines, 2 of which corresponded to the V and W lines of Burrows and Bacon (1956*a*). Crumpton and Davies showed that the 2 extra lines (called X and Y) were also present in virulent *Past. pestis* grown in 11 hr. shake cultures (Crumpton and Davies, 1956) but not in 40 hr. cultures. In some samples examined in this work only 3 lines were apparent but closer examination showed 1 line to be obscured beneath a stronger one.

US—Against unabsorbed serum, the usual spectrum of precipitation lines (Crumpton and Davies, 1956) was obtained; Fraction 1 (antigen 3) was present but not antigen 4. Against absorbed serum, only 3 lines were usually apparent 2 of which corresponded to the V and W lines of Burrows and Bacon (1956*a*) and the V line was the stronger.

R—Three lines were produced against unabsorbed serum and one of these was common with a line produced by Fraction 1. With absorbed serum only one major line occurred. The results with this material are described more fully in a subsequent paper (Keppie, Cocking, Witt and Smith, 1960).

Saline/exudate.—Against unabsorbed antiserum, undiluted exudate formed the usual complex spectrum of precipitation lines (Crumpton and Davies, 1956) but much fainter than that formed by an ultrasonic extract of its original bacterial content. Against absorbed serum, only one line—the W line—was formed. It appeared that the substance responsible for the W line was mainly an extracellular product since the line formed by the saline/exudate was stronger (approx. 2–3 times) than the W line formed by an ultrasonic extract of the original bacterial content. These results were confirmed with undiluted exudate from infected guinea-pigs.

Saline wash.—Against unabsorbed antiserum this material, when concentrated, formed only a very weak spectrum of lines. It formed only a trace of the W line against absorbed antiserum but produced a weak V line possibly as a result of washing a surface component from the organisms.

The aggressive activity of products from Past. pestis *grown* in vivo

The Table summarizes the results of numerous assays for virulence enhancing, and antiphagocytic activity with different batches of saline/exudate from infected guinea-pigs, and also US and R derived from *Past. pestis* isolated therefrom. All these crude products had aggressive activity especially R which was mainly cell wall material. In addition, Fraction 1 was active in these antiphagocytic tests with guinea-pig blood (0·02 per cent active) and mouse blood (0·01 per cent active).

TABLE.—*Virulence-enhancing and Antiphagocytic Activities of Products from* Past. pestis *grown* in vivo

Material from *Past. pestis* grown *in vivo*	Virulence enhancing activity	Antiphagocytic activities against	
		Guinea-pig cells	Mouse cells
Saline/exudate	Inactive undiluted	Active undiluted*	Trace of activity undiluted.*
US	Active undiluted	,, ,,	Active undiluted.
R (in solution)	,, ,,	Active 1/27	Active 1/4.

The results are the average of several similar results on 2–3 batches of material.

* The activity in the saline/exudate was confirmed by examining undiluted exudate and plasma collected separately from guinea-pigs.

DISCUSSION AND SUMMARY

Past. pestis (strain L37) has been obtained from guinea-pigs dying of plague in sufficient quantity (4·8–6·8 g. from 400 guinea-pigs) and of adequate purity for chemical work on the basis of its virulence and immunogenicity in guinea-pigs and mice. Large quantities of saline washings from the thorax and peritoneum of the infected guinea-pigs have been collected to provide the extracellular products of *Past. pestis* and the easily removed surface components. The organisms have been extracted with ultrasonic waves; approx. 70 per cent. of each organism dissolved leaving a residue of predominantly cell wall material.

The products of *Past. pestis* grown *in vivo* have aggressive activity because they enhance the virulence of *Past. pestis* for guinea-pigs and inhibit the phagocytosis of *Past. pestis* by leucocytes from the guinea-pig and mouse. The antiphagocytic activity of these materials must be due to their content of Fraction 1, at least to some extent, since we have confirmed the antiphagocytic activity of this fraction reported by Chen and Meyer (1954). Chemical extraction of the crude materials may reveal further bases for the aggressive activity, especially as they contain in addition to Fraction 1, the compounds forming the V and W precipitin lines, which also have been implicated in resistance to phagocytosis. Any connection between the compounds responsible for aggressive activity and those having immunogenic and toxic activity will also be investigated in future chemical extractions.

In addition to V and W, 2 further precipitinogens X and Y detectable with antiserum adsorbed with avirulent organisms, were produced by virulent organisms *in vivo* and under certain conditions *in vitro*. The relative importance of these materials in determining virulence may become clear as chemical fractionation proceeds.

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THE CHEMICAL BASIS OF THE VIRULENCE OF *PASTEURELLA PESTIS*

II. The Toxicity for Guinea-pigs and Mice of Products of *Past. pestis*

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This paper describes the toxicity of products, obtained first from *Past. pestis* grown *in vivo* (Smith, Keppie, Cocking and Witt, 1960) and then from organisms produced *in vitro*. These products killed guinea-pigs as well as mice and provided a reasonable explanation for the death of guinea-pigs from plague. Additional studies are described—including fractionation—which give some insight into the nature and interrelationship of the toxic products. Some of this work has been reported briefly elsewhere (Keppie, Smith and Cocking, 1957; Cocking and Smith, 1958).

MATERIALS AND METHODS

Extracellular products of Past. pestis *grown* in vivo.—The plasma of guinea-pigs newly dead from plague was collected as described by Smith, Keppie and Stanley (1955) for the plasma of anthrax-infected guinea-pigs. Undiluted thoracic and peritoneal exudate, saline/exudate, and the saline washes of the organisms from these exudates were obtained as described previously (Smith *et al.*, 1960).

Ultrasonic extract (US) and residue (R) of Past. pestis *grown* in vivo.—These were prepared as described previously (Smith *et al.*, 1960).

US and R, at concentrations equivalent to 1×10^{10} organisms per ml., contained respectively approx. 3 and 1 mg. of *Past. pestis* material per ml. For some experiments, a solution of R was prepared by treating it with alkali (Keppie, Cocking, Witt and Smith, 1960).

Extracts and residues prepared by treating Past. pestis *grown* in vitro *with ultrasonic waves.*—These were prepared from the virulent strain L.37 and the avirulent Tjiwidej strain of *Past. pestis* grown for 24 hr. at 28° and 37° in the medium of Davies (1956) and a modification of that of Higuchi and Carlin (1957) (Keppie *et al.*, 1960). The extracts and suspensions of the residues were used at concentrations equivalent to 1×10^{10} organisms per ml.

Lipopolysaccharide of Past. pestis (*Tjiwidej*).—We are indebted to our colleague Dr. D. A. L. Davies for this material.

Purified "mouse toxin" from culture in vitro.—We are indebted to our colleague Dr. A. P. MacLennan for this material (approx. LD_{50} for mice—0·2 μg. by intravenous injection).

Chromatography on diethylamino ethyl-cellulose (DEAE-C), (*Peterson and Sober,* 1956).—Before use, DEAE-C (Eastman Kodak Ltd.) was regenerated with 1·0 N NaOH and equilibrated with 0·02 M tris (hydroxy methyl) amino-methane hydrochloride (THAM-HCl) buffer at pH 7·5. Freeze dried US (200 mg.) in 0·02 M THAM-HCl buffer (3 ml.) at pH 7·5 was added to a column (glass, 1·5 cm. diam.) of DEAE-C (3 g.) and stepwise eluted (flow-rate 60 ml./15 min.) with buffers of constant pH (7·5) but increasing molarity (0·1 M, 0·2 M and 0·5 M, THAM-HCl); all work was done at + 4°. As a measure of eluted material the extinction of light (280 mμ) by fractions (2 ml.) was determined in a Unicam Spectrophotometer (½ cm. cell).

Chromatography on hydroxylapatite (HA; Tiselius, Hjerten and Levin, 1956).—HA was prepared as described by Tiselius *et al.* (1956) except that the final product was stored

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at room temperature in 0·01 M phosphate buffer at pH 6·8. Chromatography on columns of HA (3 g.) was similar to that using DEAE-C and the loading is described in the text. The columns were eluted (flow rate 60 ml./hr.) stepwise with phosphate buffers (0·01 M, 0·1 M and 0·2 M) of constant pH (6·8).

Concentration of toxic fractions by dialysis against carbowax (poly-ethylene glycol. 20 M).—Since the toxicity of some fractions was destroyed by freeze drying, samples from chromatographic columns were concentrated by the method of Kohn (1959). No leakage of carbowax through the Visking dialysis tubing could be detected either from optical density measurements at 280 mμ or from estimations of the intensity of pink colour produced when aqueous carbowax reacts with A.R. H_2SO_4 (diluted with 1/6th of its volume of water).

Serological precipitation in gel diffusion plates.—The technique and antisera used were described previously (Smith *et al.*, 1960).

Toxicity tests

Lethality for mice (20–22 g.).—Mice were injected intravenously and observed for 7 days.

Lethality for guinea-pigs (250 g. $\pm$ 25 g.).—Guinea-pigs were injected intravenously by the method of Smith, Keppie and Stanley (1955) and observed for 7 days.

Production of oedema.—The shaven flanks of guinea-pigs (500 g.) or rats (350 g.) were injected with dilutions of the material (0·2 ml.). After 18 hr. the highest dilution of the material still producing oedematous reaction was noted.

Bacterial counts on infected tissue, blood and peritoneal washes.

Blood and other infected fluids were suitably diluted and their content of *Past. pestis* was counted by haemocytometer. Weighed amounts of the several infected tissues were finely ground with sand and saline in a mortar, and the bacterial content for each organ was similarly calculated from haemocytometer counts. When viable counts were done, these approximated closely to the direct counts.

EXPERIMENTAL

Evidence that No Purely Extracellular Toxin was Produced In Vivo

Blood, containing 1–5 $\times$ 10^9 organisms per ml. was collected from guinea-pigs dying from plague. It was cooled, and the blood cells and bacteria immediately removed from the plasma. The plasma did not kill guinea-pigs (10 ml. injected intravenously) and was only weakly lethal for mice (LD_{50} approx. 0·5 ml. given i.v.). This slight toxicity was probably due to liberation of intracellular toxin since it was small in comparison with that of an appropriate extract of the organisms originally present (see below), and was much increased (LD_{50} approx. 0·05 ml.) either by delaying the original collection of blood until 1 hr. after death or by storing the plasma unfiltered at 0°.

Neither the peritoneal and thoracic exudates nor the saline exudate or saline wash of the *Past. pestis* obtained from infected guinea-pigs contained significant amounts of toxin in comparison with that found in the disintegrated organisms.

The lethality for guinea-pigs and mice of fractions R and US from Past. pestis *grown* in vivo

Table I shows the results of toxicity tests with whole organisms, grown *in vivo*, and with corresponding US and R fractions. The toxicity of R for mice was approx. 10 per cent of that of whole organisms and was low compared with that of US; the latter was 2–4 times more toxic than the whole organisms. R (LD_{50} approx. 15·0 $\times$ 10^{10} organisms) was about half as toxic for guinea-pigs as US (LD_{50} approx. 7·5 $\times$ 10^{10} organisms). These separate toxicities of R and US for guinea-pigs did not account for that of the intact organisms (LD_{50} approx.

2×10^{10}). However mixtures of R and US appeared to show some synergic action and as a result approx. 50 per cent of the toxicity of whole organisms could be recovered. Thus 3 guinea-pigs died from doses of US + R each equivalent to 5×10^{10} organisms and 2 died from doses equivalent to 3×10^{10} *Past. pestis*. Further evidence is given later for the possibility of synergic action between fractions of US and R.

TABLE I.—*The Lethality for Guinea-pigs and Mice of* Past. pestis *Grown* In Vivo *and its Products Obtained after Treatment with Ultrasonic Waves*

Material injected	Deaths / Number injected															
	Guinea-pigs					Mice										
	20*	10	5	2·5	1·25	1·6*	0·8	0·4	0·2	0·1	0·05	0·025	0·01	0·005	0·0025	0·00125
Organisms†	—	2/2	6/6	18/18	1/16	—	—	—	—	—	20/20	20/20	13/20	9/20	0/20	0/20
US	6/6	10/12	2/10	0/3	—	—	—	—	—	—	—	—	20/20	68/71	70/104	24/68
R																
(*a*) Untreated†	6/6	2/14	0/3	—	—	5/5	5/5	5/5	10/10	13/13	6/13	4/13	—	—	—	—
(*b*) In solution after treatment with alkali‡	5/6	4/15	0/4	—	—	8/10	1/10	0/10	—	—	—	—	—	—	—	—

* Quantities relate to the number ($\times 10^{10}$) organisms injected or from which the product was obtained.

† The suspension of organisms (or R) was injected with 0·02 mg./ml. streptomycin. Mice received 4 mg. streptomycin subcutaneously prior to injection of organisms and 4 mg. later in the day, and thereafter 4 mg. per day for 1 week. Guinea-pigs received 80 mg. streptomycin subcutaneously prior to injection of organisms, 40 mg. later in the day and thereafter streptomycin cerate (0·02 g. intramuscularly) daily for 1 week. It is impossible to say that no growth or metabolism of the organisms took place before their destruction by the streptomycin. However deaths occurred within 2 days of injection and postmortems on dead animals showed the latter died in the absence of infection.

‡ See Keppie *et al.*, 1960.

Rapid destruction of toxicity for mice but not that for guinea-pigs by a short alkaline treatment

Treatment with alkali rapidly reduced the toxicity of R for mice (Table I). Similarly, the LD_{50} of US and whole organisms for mice was raised to 0·1 ml. by treatment with N/10 NaOH at 18° for 5 min. followed by adjusting the pH to 9·2 with glycine. On the other hand, the toxicity of R, US or whole organisms for guinea-pigs was not reduced by a similar treatment with alkali.

The number of Past. pestis *organisms present in guinea-pigs and mice when dying from plague*

The results in Table I indicate that sufficient toxin to kill a guinea-pig (250 g.) can be obtained from $2{\cdot}5 \times 10^{10}$ *Past. pestis* organisms. If the demonstration of this toxicity was to provide a reasonable explanation of death of guinea-pigs from

plague, guinea-pigs dying from infection should contain or have contained at least $2{\cdot}5 \times 10^{10}$ organisms. The following experiments indicated that this was so.

Guinea-pigs (250 g.) were infected either intraperitoneally or subcutaneously with *Past. pestis* (L.37, 1×10^8 organisms). When dying 4–7 days later they were killed and the plague bacilli in the major tissues were counted. The results shown in Table II (which accord well with similar results using larger guinea-pigs) indicated that the guinea-pigs contained at least $6–16 \times 10^{10}$ *Past. pestis*.

TABLE II.—*Number and Distribution of* Past. pestis *in Guinea-pigs Dying from Plague*

	Bacterial content ($\times 10^{10}$) of tissues of dying guinea-pigs (250 g.)			
	Infected intraperitoneally		Infected subcutaneously	
	(1)	(2)	(1)	(2)
Blood	1·3	1·5	0·9	2·4
Peritoneum	4·8	8·0	0·5	0·6
Liver	0·9	5·2	2·2	1·9
Spleen	0·7	0·8	2·6	0·4
Lungs	0·1	0·4	0·4	0·3
Kidneys	0·1	0·2	0·1	0·1
Total ($\times 10^{10}$)	7·9	16·1	6·7	5·7

Similar budgets for *Past. pestis* in mice dying from plague showed that they contained at least $5–7 \times 10^9$ organisms—a number which was adequate for providing more than one lethal dose of the mouse toxin (Table I).

Pathological changes produced in guinea-pigs and mice by plague infection, and by toxic products of Past. pestis *grown* in vivo

Guinea-pigs and mice died from plague with the clinical signs of shock. When either species was injected intravenously with a lethal dose of US or R from *Past. pestis* grown *in vivo* they became ill within a few hours and died with the clinical signs of shock usually within 24 hr. and always within 48 hr. At post-mortem, both infected and toxic animals showed congestion of the blood vessels and some oedema but there was no widespread and extensive oedema comparable to that found in anthrax and in infection with say *Clostridium oedematiens*.

We have not carried out an extensive chemical investigation into the nature of the shock syndrome in infected and toxaemic animals, comparable to the study of anthrax (Smith, Keppie, Stanley and Harris-Smith, 1955). Our colleague Dr. M. Lancaster has made the following observations on the histo-pathology which showed that lesions in infected and toxaemic animals were similar, and although oedema was slight, general venous congestion was marked and probably contributed to the shock syndrome. The only other major pathological change was scattered focal necrosis in the liver.

Infection in guinea-pigs

The subcutaneous injection of a lethal dose of strain L.37 results in general venous congestion (particularly of the mesenteric vessels), some exudation into

the body-cavities, and visceral lesions in the liver, spleen, adrenals and intestines. The liver has small foci of necrosis, and histological examination of these reveals well-marked centrilobular fatty change, capillary congestion, and scattered micro-abscesses which consist of bacteria surrounded by necrosis and polymorph infiltration. The Kupffer cells are crowded with organisms. The irregular staining of organisms in some of the clumps indicates lysis. The bright pink adrenals are found to have general capillary congestion with haemorrhages in the cortex, while the cells of the mid cortical zone show hydroptic changes, vacuolation, necrosis, and some overall diminution of cortical lipid. The adrenal changes are found in animals prior to death and are the first visceral lesions to appear. The spleen is congested and contains a variable number of bacteria, also foci of necrosis and inflammatory cell infiltration. The kidneys show slight tubular damage—however the staining for alkaline phosphatase is normal. No damage was found in the myocardium or in the CNS.

Toxaemia in guinea-pigs

In guinea-pigs, the changes produced by the intravenous injection of the toxic extract (US) are similar to those following infection.

Venous congestion is marked both in the mesentery and intestines, and the other main changes are found in the liver, adrenals, and lung. The liver is congested, and shows early centralobular fatty change, loss of cell basophilia, and loss of glycogen. Eosinophilic cytoplasmic inclusions are found. They are circular, vary in size, and stain positively with PAS, with Masson trichrome and Phostox. These inclusions appear to be similar to those found in some types of hepatitis, and probably represent the passage of plasma into the damaged liver cells (Nairn, Chadwick and McEntegart, 1958). Scattered single necrotic cells are found, often with surrounding nuclear debris ; however the guinea-pig does not show the necrotic foci and polymorph infiltration seen in mice. The changes in the adrenals, kidneys, and CNS are as described under " Infection ". The intestines may show small submucosal haemorrhages, and there are often massive haemorrhages into the lumen of the stomach and intestines.

Toxaemia and infection in mice

The lesions resulting from injecting mice with US were similar to those caused by infection and also to the findings of earlier workers (Schar and Meyer, 1956). Briefly the liver showed scattered micro-abscesses with variable leucocyte-infiltration but no inclusion bodies. The adrenals only showed slight congestion and the tubular damage in the kidneys was slight.

Toxaemia in rats

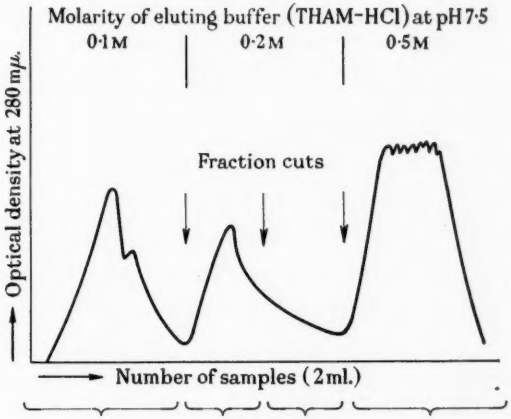
In rats, US also produced lesions similar to those found in mice. In addition, however, the damaged liver cells occasionally contained cytoplasmic inclusions as in guinea-pigs.

Preliminary Fractionation of US

To economise in material expended on toxicity tests, US was fractionated and at first tested only in mice. When some success had been achieved, fractions which were limited in quantity were examined for toxicity in a few guinea-pigs.

Chromatography on DEAE-C : synergic action of fractions in toxicity for guinea-pigs

US (430 ml.) was dialysed against distilled water (15 l.) at 4° overnight and freeze dried. Freeze dried US (200 mg. ≡ approx. 60 ml. original extract) was chromatographed on DEAE-C as described in "Methods". The Fig. summarises



	Original US (200 mg.)	A	B	C	D
Measure* of mouse toxin	16,000‡	Neglig.	750	8,750	5,000
Measure† of protein content	450	66	19	15	330

* The LD_{50} of the mouse toxin was determined and the number of LD_{50} in the total fraction was calculated; this figure is quoted. The results are representative of numerous assays (involving in all 25–50 mice for each dilution of each fraction) on the same fractions from several chromatograms.

† The extinction of light at 280 mμ was measured at a suitable dilution (see "methods") and multiplied by the dilutions involved to give the quoted figures for the whole fraction.

‡ Before dialysis and freeze drying the figure was 24,000.

FIG.—Chromatography of US on DEAE-C; mouse toxicity of fractions.

the results of chromatography in which 4 fractions (A, B, C and D) were collected and the total recovery of toxin was satisfactory. Toxicity was concentrated in fraction C which had an approx. LD_{50} for a mouse of 0·73 μg. as compared with 12·5 μg. for freeze dried US (assuming that the same relationship between optical density and dry weight existed for both mtaerials). Practically all the original nucleic acid was present in fraction D. Serological precipitation on plates showed the envelope antigen of Baker, Somner, Foster, Meyer and Meyer (1952) and other antigens to be present in all fractions, but V and W antigens were concentrated in fractions from the leading edges of the 0·2 M and 0·5 M THAM-HCl elution peaks respectively.

Fractions A, B, C and D were now tested for toxicity in guinea-pigs. Five batches (200 mg.) freeze dried US (≡ approx. 300 ml. original extract and approx. 30–40 LD_{50} of toxin for guinea-pigs—see Table I) were chromatographed as above. Before injection individual fractions were bulked and concentrated by dialysis against carbowax. All four fractions were non-toxic for guinea-pigs when injected alone in large amount (see Table III). After preliminary tests had shown that a mixture of all 4 fractions was toxic for guinea-pigs and titration of this mixture had indicated a satisfactory recovery of total guinea-pig toxin, various mixtures of 2 fractions were tested for toxicity (see Table III). Fraction A contained one component of an apparently synergic mixture, and fractions C and D the other. As might be expected, fraction B appeared to contain a little of both components.

As noted above, amounts of R and US which were non-toxic when injected alone, were toxic if injected together. If US was in turn replaced by each of the 4 fractions described above, only the mixture with fraction A was toxic (see Table III). This indicated that R contained an excess of the component of the guinea-pig toxin which in US fractionates into fractions C and D.

Purification of the mouse toxin by chromatography on HA

Chromatography of fraction C on HA removed the envelope antigen selectively from the mouse toxin possibly because separation of proteins on HA is less dependent on differences in isoelectic points than is chromatography on DEAE-C (Tiselius, *et al.*, 1956).

Fraction C (from dialysed and freeze dried US 200 mg.) from the DEAE-C column was diluted with water until the THAM-HCl was 0·04 M and added to a column of HA (3 g.). After elution with 0·01 M, 0·1 M, and 0·2 M phosphate buffer at pH 6·8 as described in " Methods ", all the mouse toxin was present in the one elution peak removed by the 0·1 M buffer. The envelope antigen and other antigens had been largely removed by elution with 0·01 M buffer and other non-toxic antigens remained on the column to be removed by the 0·2 M buffer.

The fraction rich in mouse toxin was almost free of envelope antigen (when compared on gel diffusion plates with fraction C); toxicity assays and light absorption measurements at 280 mμ indicated that a fourfold concentration of mouse toxin (relative to fraction C) had been achieved, and that its LD_{50} for a mouse was approx. 0·2 μg. (assuming the same relationship between optical density and dry weight as existed for freeze dried US). In two experiments, three successive portions of fraction C from a DEAE-C column were chromatographed separately on HA (3 g.). These experiments indicated that an even greater purification of the mouse toxin could be obtained with a lower column loading, and that rechromatography on HA of the active fraction removed further small amounts of envelope antigen.

The fractions from HA containing mouse toxin formed in serological diffusion plates against plague antiserum, one main line and two faint ones. The main line in these plates was associated with mouse toxicity since it was also formed by a purified sample of mouse toxin from *Past. pestis* grown *in vitro* (see " Methods ") and was not formed by samples rendered relatively non-toxic by treatment with alkali. The most purified samples produced in a guinea-pig an antiserum which did not precipitate the envelope antigen and formed only one serological precipi-

tation line with US, and the purified preparations of mouse toxin from *in vivo* and *in vitro* sources.

In toxicity tests in guinea-pigs comparable to those shown in Table III our purified mouse toxin (like its parent fraction C) did not kill guinea-pigs when injected alone at the high dosage (Table III) but did so (2) at the low dosage when mixed with fraction A. Furthermore when an amount ≡ 5–10,000 mouse LD_{50} was mixed with a solution of R (≡ 5×10^{10} *Past. pestis*) the mixture killed guinea-pigs (2). In contrast, if our purified mouse toxin from *in vivo* sources was replaced in the latter mixture by purified mouse toxin from *in vitro* sources (20,000 mouse LD_{50}) no guinea-pigs died.

TABLE III.—*Toxicity of Mixtures of Fractions from US for Guinea-pigs*

Injection solutions	Proportion of yield injected* (per cent)	Number dead / Number in batch A	B	C	D
Each fraction alone	50	0/2	0/2	0/2	0/2
	25	0/1	0/1	—	0/1
Mixtures of fraction A with each of other fractions	10	—	0†/2	2/2	4/4
Mixtures of fraction B with each of other fractions	10	0/2	—	2/4	0/2
Mixtures of fraction C with each of other fractions	10	2/2	2/4	—	0/2
Mixtures of fraction D with each of other fractions	10	4/4	0/2	0/2	—
Mixtures of residue‡ R. in solution (≡ 2×10^{10} *Past. pestis*) with each fraction	10	2/2	0/2	0/2	0/2

* Derived from US ≡ 30–40 guinea-pig LD_{50} (see Table I).

† If the proportion of fraction B was increased to 25 per cent, then the mixture with A killed guinea-pigs $\left(\frac{2}{2}\right)$.

‡ This amount of residue was much smaller than that required to kill guinea-pigs when injected alone (see Table I).

Oedema production in Guinea-pigs and Rats by the Toxic Products from Past. pestis *Grown* In Vivo *and by Purified "Mouse Toxin" from Culture* In Vitro

Table IV shows the results of tests for oedema production in rats and guinea-pigs using US, R, and the 2 preparations of purified mouse toxin; the minimal reactive doses have been quoted in terms of approx. LD_{50} for mice and guinea-pigs.

US and R produced oedematous lesions in rats and mice but in agreement with the mild oedema in infected and toxaemic animals previously noted, their reactions were less marked than that formed by comparable amounts (based on LD_{50} for mice and guinea-pigs) of crude anthrax toxin. The results in Table IV

show that the ability of US and R to produce oedema in rats and guinea-pigs paralleled their respective toxicity for guinea-pigs rather than their toxicities for mice. These results are supported by the fact that the ability of mixtures of US and R or whole organisms (with streptomycin) to produce oedema in rats and guinea-pigs was not reduced by the short treatment with alkali which is described above and which destroys the toxicity for mice but not that for guinea-pigs.

The tests for oedema production in guinea-pigs showed a difference between the two forms of mouse toxin (*i.e.* from *in vivo* and *in vitro* sources) and between them and US. The minimal reacting dose of the mouse toxin from *in vitro* source was more toxic (15 ×) for mice than the corresponding dose of the mouse toxin from *in vivo* sources; this latter dose was in turn 10 times more toxic for mice than the minimal reacting dose of US.

TABLE IV.—*Oedema Production in Guinea-pigs and Rats by the Toxic Products from* Past. pestis *Grown* In Vivo *and Purified "Mouse Toxin" from Culture* In Vitro

Material	Minimal reacting doses in test for production of oedema* in			
	Guinea-pigs		Rats	
	As approx. No. LD_{50} for guinea-pigs	As approx. No. LD_{50} for mice	As approx. No. LD_{50} for guinea-pigs	As approx. No. LD_{50} for mice
US	0·003	10	0·003	10
R (solution)	0·002	0·03	0·002	3
Purified mouse toxin (*in vivo*)	—	100	—	10
Purified mouse toxin (*in vitro*)	—	1500	—	10

* For details of tests see "methods".

Demonstration of Toxicity for Guinea-pigs and Mice in Products of Past. pestis *grown* In Vitro

A virulent (L.37) and an avirulent strain (Tjiwidej) of *Past. pestis* were grown under a variety of conditions *in vitro* and extracted by treating them with ultrasonic waves (see "Methods"). Only the soluble extracts were examined for toxicity and the results are shown in Table V. A guinea-pig toxin as well as a mouse toxin was produced under these conditions. When an extract of *Past. pestis* (Tjiwidej) was fractionated on DEAE-C as described for US similar elution peaks were obtained and there were indications of a similar synergic effect between fractions in toxicity tests with guinea-pigs.

DISCUSSION

In vivo, *Past. pestis* does not appear to produce an extracellular toxin comparable with that elaborated by *Bacillus anthracis*. On the other hand, it has been shown that products of the organisms (grown *in vivo* or *in vitro*) obtained by ultrasonic disintegration are toxic for guinea-pigs as well as mice. The yield of toxin was such as to provide a reasonable explanation for death of both species as a result of infection, because at death, infected animals had a bacterial population greater than that necessary to provide one lethal dose of toxin. Since only a trace of free toxin could be detected in freshly collected and processed body fluids, this explanation for death would hold only if the toxin was liberated

in vivo and then immediately fixed by tissues as in the case of some clostridial toxins.

When injected with toxic preparations, guinea-pigs and mice died with the clinical signs of shock (as did infected animals) but there was no extensive oedema comparable to that occurring in anthrax. Furthermore, in intradermal skin tests for oedema the toxic products of *Past. pestis* were less active than samples of the anthrax toxin of comparable lethality for mice and guinea-pigs. The question arises therefore whether the shock syndrome is due to a gross leakage of plasma fluid (Schar and Meyer, 1956) or to some other cause, *e.g.* pooling of peripheral blood, etc.

TABLE V.—*Toxicity for Guinea-pigs and Mice of Ultrasonic Extracts of* Past. pestis *Grown* In Vitro

Culture from which ultrasonic extract made			Mice (ml.)*			Guinea-pig (ml.)*		
Strain	Growth temperature (°)	Medium	0·005	0·0025	0·00125	15	10	5
Virulent								
L37	28	Davies (1956)	10/10	10/10	13/13	—	7/7	6/10
	28	Higuchi and Carlin (1957)	11/16	5/8	0/4	1/1	3/3	2/4
Avirulent								
Tjiwidej	28	Davies (1956)	13/16	11/14	2/10	—	4/4	—
	37	,, ,,	10/10	23/28	7/13	11/12	4/12	1/4
	28	Higuchi and Carlin (1957)	8/16	3/6	—	1/1	1/2	—

* Strength of ultrasonic extract ≡ 1×10^{10} *Past. pestis* per ml.

An experiment with live organisms and streptomycin comparable to that shown in the first line of Table I but using Tjiwidej organisms, showed the LD_{50} of whole organisms for guinea-pigs (250 g.) and mice were approx. 2×10^{10} and 1×10^{8} respectively.

The nature of the native toxin or toxins is still unknown. When organisms were disrupted with ultrasonic waves, the toxicity for mice and guinea-pigs was divided between the soluble extract and the residue—which was largely cell wall material and contained practically no intact organisms. The toxicity for mice was predominantly in the extract while some remained with the residue, but in contrast the toxicity for guinea-pigs was only slightly more in the extract than in the residue. This raises the question of whether the toxins demonstrated were incomplete portions of a larger complex occurring in the intact organisms. There are some indications that this might be so from the results of budgets for toxicity in the initial extraction of *Past. pestis* (Table I) and during the fractionation of the ultrasonic extract.

The mouse toxin was more potent when liberated in the ultrasonic extract than when fixed in intact organisms (see Table I). The fraction containing the mouse toxin from organisms grown *in vivo* was at least as potent as previous

preparations obtained from *Past. pestis* grown *in vitro* (Ajl, Reedal, Durrum and Warren, 1955; Ajl, Rust, Hunter, Woebbe and Bent, 1958; Spivack and Karber, 1958; Pillemer and Robbins, 1949). The two types were serologically related but not identical. Thus, the purified preparation from *in vivo* source, formed more oedema in the skin of a guinea-pig than a comparable dose (based on mouse LD_{50}) of the material from *in vitro* sources. Again, our preparation killed guinea-pigs when mixed with the ultrasonic residue, R, whereas the one from artificial culture did not. It appears therefore that the former contained a residual ability to be toxic for guinea-pigs whereas the latter did not.

The sum of the separate toxicities of the ultrasonic extract and residue for guinea-pigs did not account for the toxicity of the whole organisms (Table I). This anomaly was explained by the fact that guinea-pig toxicity appeared to be due to synergic action of at least two components. These were present in the crude products after treatment of *Past. pestis* with ultrasonic waves but may have been a single complex in the intact organism. When the ultrasonic extract was fractionated on DEAE-C columns the components of the synergic mixture were separated. One was in a fraction devoid of mouse toxicity whereas the other was present in fractions with high mouse toxicities. Although both components were impure, the results might suggest that the mouse toxin is a component of the guinea-pig. toxin. However, this appears to be ruled out by the fact that the residue (in solution after treatment with alkali which destroys the residual mouse toxicity) could replace in the synergic mixtures the fractions containing the mouse toxin. The residue (R) contained predominantly one component (see Table IV) of the synergic mixture but it also contained some of the other component since it was toxic when injected alone in large amount ($\equiv 20 \times 10^{10}$ *Past. pestis*).

It is possible that the guinea-pig toxin or one component of it contained in its structure the lipopolysaccharide isolated by Davies (1956) after degrading the products of *Past. pestis* with hot phenol. This polysaccharide was toxic to guinea-pigs but its toxicity (LD_{50} approx. 2 mg. i.v. after treatment with alkali as described for R) *per se* did not account for the toxicity of R (LD_{50} approx. 15 mg.; lipopolysaccharide content approx. 2·6 per cent—see Keppie, Cocking, Witt and Smith, 1960). Furthermore the polysaccharide (0·1 mg.) did not replace any component of the toxic mixtures described in Table IV.

The nature of the components of the guinea-pig toxin and their possible connection with the mouse toxin can only be ascertained by further chemical purification. It may be that an even milder extraction of *Past. pestis* than the present treatment with ultrasonic waves will yield a more complete toxin with the synergic components combined.

SUMMARY

An explanation for the death of guinea-pigs from plague has been provided by the preparation of products which kill a guinea-pig from numbers of *Past. pestis* which are less than those present in a guinea-pig when it died of plague.

The toxic products which also killed mice, were first obtained from *Past. pestis* grown *in vivo* by treatment with ultrasonic waves. Toxicity was divided between the soluble extract and residue.

Material toxic for mice was predominantly in the extract and was considerably purified by chromatography first on diethylamino ethyl cellulose (DEAE-C) and then on hydroxyl apatite (HA) which selectively removed envelope antigen.

The toxicity for guinea-pigs was due to a mixture of two components which were unequally divided between the residue (mainly cell wall material) and the extract. These components were separated from the extract by chromatography on DEAE-C but as yet, are of unknown constitution.

The possibility exists that the products as prepared and fractionated were fragments of a more complete toxin.

Similar products were obtained from *Past. pestis* grown *in vitro*.

Toxaemic animals died with the clinical signs of shock as did infected animals. The formation of oedema by the products was not sufficiently large to indicate that this was the primary cause of the shock but general venous congestion and liver damage might contribute to the latter.

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THE EFFECT OF TRITON, A SURFACE ACTIVE POLYOXEYTHYLENE ETHER, ON EXPERIMENTAL INFECTIVE PNEUMOCONIOSIS

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IN a recent paper (Byers, King and Harrison, 1960) a lack of effect of Triton on quartz-induced silicosis was described. Previous work from this laboratory has demonstrated that a condition resembling the progressive massive fibrosis of coal workers and kaolin workers could be induced in animal lungs by the combined action of dust with different forms of tubercle bacilli (Attygalle, Harrison, King and Mohanty, 1954; Attygalle, King, Harrison and Nagelschmidt, 1956; King, Yoganathan, Harrison and Mitchison, 1957; Zaidi, Harrison, King and Mitchison, 1955). In view of the work of Rees (1953) and of Hart and Rees (1955) on the prevention of tuberculosis in mice by Triton, it was thought worthwhile to determine whether it had any effect on the highly modified form of tuberculosis induced by a mixture of a large amount of dust with a relatively small number of tubercle bacilli in guinea-pigs and rats, and which we have called infective pneumoconiosis. One of the organisms used was the vole bacillus *Mycobacterium tuberculosis* (var. *muris*) described by Wells (1937). Experimental infective pneumoconiosis has already been produced by this organism combined with coal-mine dust by King *et al.* (1957). B.C.G. had similarly been used with kaolin, with a positive result (Attygalle *et al.*, 1954). We had previously failed to show any lessening of the severity of the lesions produced by coal dust combined with a photochromogenic bacterium when the animals were treated with Triton (Byers and King, 1959), but this organism was not of the tuberculosis group and it was therefore thought worthwhile to assess the effect of Triton on a strain of *Myco. tuberculosis* whose activity in experimental infective pneumoconiosis was already known. At the same time it was necessary to confirm the opinion that Triton would have no effect on the lesions produced by coal and kaolin alone.

MATERIALS AND METHODS

Experiments.—Ninety-four male albino guinea-pigs of the Hartley strain were divided into groups as shown in Table I and injected with the following mixtures: Vole bacillus, vole bacillus with Triton, coal dust plus Triton, coal dust plus vole bacillus, coal dust plus vole bacillus plus Triton, kaolin plus triton, kaolin plus vole bacillus and kaolin plus vole bacillus plus Triton.

Groups of animals treated with coal alone and kaolin alone are not included, since we had repeatedly found that these dusts produced minimal fibrosis (less than Grade 1) in the lungs of guinea-pigs. It was planned to kill animals at monthly intervals and to assess the results by gross and microscopic examination of the lungs.

Dusts.—The artificial anthracite coal-mine dusts described by King *et al.* (1957) and Zaidi *et al.* (1955) and the kaolin used by Kettle (1934) and by Attygalle *et al.* (1954) and made by British Drug Houses to the specification of the Leather Trades Society were used.

Mycobacterium tuberculosis var. *muris.*—Dr. D. Weiss of the Sir William Dunn School

of Pathology, Oxford, kindly supplied us with cultures of this organism. The first culture was found not to be viable : there was a complete absence of lesions at 4 weeks in control animals and an absence of growth on solid medium. A second supply of the organism was found to be viable and from this a suspension of organisms was made for re-injecting the animals.

Suspensions for injection.—The original vole bacilli were supplied in a known concentration of 10^6 organisms/ml. in 0·9 per cent saline. This was suitably diluted with sterile saline, or with dust suspended in saline (and sterilized), to give $6{\cdot}6 \times 10^3$ organisms/ml. Viable counts were made according to the technique of Selkon and Mitchison (1957).

The dust suspensions were made in concentrated form such that when used to dilute the vole bacillus a final concentration of 66 mg./ml. of dust was obtained. When used alone the dust was diluted with sterile saline.

The second injection of vole bacillus was from organisms subcultured on Middlebrook-Dubos-tween-albumin liquid medium without glycerol. The concentrated solution was diluted to 10^4 organisms/ml., and viable counts were performed. By counting the colonies from suitably diluted suspensions, $3 \pm 0{\cdot}9 \times 10^3$ organisms/ml. were found.

Injection technique.—The technique described previously (Byers *et al.*, 1960) was employed. Anaesthesia was with ether. The volume of dust, of organisms, or of dust plus organisms was 1·5 ml. The second injection of organisms was of 1 ml.

Killing, autopsy and fixation.—The animals were killed by coal gas and the lungs inflated *in situ* with 10–15 ml. of 10 per cent formalin. Sections were cut at 5–7 μ, and stained with haematoxylin and eosin, silver impregnation (Gordon and Sweet, 1936) for reticulin and Ziehl-Neelsen for mycobacteria.

Triton solution for treatment.—The Triton solution used, Triton WR.1339 of the Bayer Company, was a 12·5 per cent aqueous solution, and twice weekly injections were given of 125 mg./kg.

RESULTS

There were 5 deaths after the first injections ; 3 perforations of the oesophagus, 1 acute pneumonia, 1 rupture of the liver. After the second injection, 68 days after the first, there were no immediate deaths, but one animal died at each of 2 and 3 weeks in the kaolin plus vole plus Triton group. In these two animals and in two at the 98 day killing (Table I) a pseudo-tuberculosis infection was found affecting the lungs. A total of 16 cases of pseudo-tuberculosis was found in these and in subsequent deaths and killings. Because of the danger to the other guinea-pigs and the stock it was decided to terminate the experiment at 135 days, *i.e.* 67 days after the second injection of vole bacilli. The Triton-treated coal and kaolin-injected animals (without vole bacillus) were likewise killed at 135 days. The deaths and killings are set out in Table I.

Neither coal dust nor kaolin caused any more than a minimal fibrotic reaction, and the Triton injections appeared to have no effect. The coal dust was phagocytosed and brought into foci around respiratory bronchioles. The kaolin tended to lie mainly in the central parts of the lung and to be engulfed in large multinucleated cells which packed the alveoli. Small outlying deposits were formed around respiratory bronchioles. There was a very small amount of reticulin in the coal foci [Grade 1 minimal in Belt and King's (1945) classification of fibrosis], and with kaolin a rather larger amount (Grade 1 maximal).

Recognizable tuberculous lesions were seen in the lungs of an animal of the vole group at 30 days and again at 67 days after the second injection. These were about 2 mm. in size, composed of epithelioid cells with a rim of lymphocytes. Fibroblasts and occasional polymorphs were present, and a few bacilli were found. No lesions due to vole bacilli were seen in the vole-injected Triton-treated animals.

In the animals injected with coal dust and vole bacillus there were lesions at 98 days in both the untreated and Triton-treated animals. (Although pseudo-

TABLE I.—*Effect of Triton on the Lung Fibrosis Produced in Guinea-pigs Injected with Dust and/or* Myco. tuberculosis *var.* muris

Dust and/or vole bacillus	Triton treatment	Days										
		0–30	30	60	68	82	89	90	98	120	128	135
Vole bacillus	−	..	(1K) 0	..		..	..	..	(1K) 1	..	(1K) 2	(5K) 2
Vole bacillus	+	..	(1K) 0	..		..	..	..	(1K) 0	..	(1K) 0	(5K) 0
Kaolin	+	..	(2K) 1 −	(1K) 1 −		..	..	(1K) 1 −	..	(1K) 1	..	(3K) 1
Kaolin and vole bacillus	−	..	(2K) 1 −	..	(Second vole injection)	(1D)	(1D)	..	(2K) 1 +	..	(2K) 2	(12K) 2
Kaolin and vole bacillus	+	..	(2K) 1 −	..		..	..	..	(3K) 1 +	..	(2K) 1 +	(1K) 0 (2K) 1 (6K) 1 +
Coal	+	(1K) 0	(1K) 1	(1K) 1 +		..	..	(1K) 1 + (1D) 1 +	..	(1K) 1 + (1D) 1 +	..	(1K) 1 +
Coal and vole bacillus	−	(1D)	(1K) 1	..		..	..	..	(2K) 1 +	..	(1K) 1 +	(1K) 1 + (2K) 2
Coal and vole bacillus	+	(3D)	(2K) 1	..		..	..	..	(1K) 1 + (1D) 1 +	..	(2K) 2	(7K) 1 + (2K) 2

The numbers and letter, K or D, in parentheses refer to the numbers of animals killed or dead at the indicated time, the following number to the grade of fibrosis found in the lungs, + signifying maximal within the grade of fibrosis and − minimal; thus (2K) 1 − means: 2 killed animals, both with grade 1 minimal fibrosis.

tuberculosis infection in some animals made it difficult to make precise assessments of the lesions, it was nevertheless possible to draw fairly confident conclusions.) Microscopically the lesions were composed of epithelioid cells, lymphocytes and monocytes, together with dust-containing cells. In the untreated animals necrosis was present, but was absent from the Triton-treated group. Small numbers of vole bacilli were found in the untreated animals, but not in those treated with Triton. At 128 and 135 days the lesions consisted of loose tangles of reticulin with some collagen. There was no discernible difference in the amount of maturity of the fibrous tissue in the untreated and Triton-treated animals.

In the kaolin plus vole bacillus groups the lesions in the Triton-treated animals were, on the whole, less extensive than in the animals untreated with Triton. But some animals in the Triton-treated group had lesions which were as extensive as those in the untreated animals. There was necrosis in the leisons of the untreated group, but not in the group receiving Triton. Small numbers of vole bacilli were present in the former but not in the latter. Reticulin fibres were present in the lesions in both groups, and occasionally formed denser tangles in which collagen fibres occurred.

TABLE II.—*Summary of the Effects Produced in the Lungs of Untreated and Triton-treated Guinea-pigs by Vole Bacillus Alone and Combined with Coal and Kaolin Dusts*

Dust and/or vole bacillus	Triton treatment	Size of lesions (mm.)	Necrosis	Cavitation	Fibrosis	Bacteria	Progression and/or regression
Vole bacillus	—	0·5–1	O	O	2	+	R
Vole bacillus	+	—	O	O	O	O	R
Kaolin + vole bacillus	—	3—C	+	O	2	+++	P
Kaolin + vole bacillus	+	2—C	O	O	1–2	O	P and R
Coal + vole bacillus	—	2–5	+	O	1–2	++	P
Coal + vole bacillus	+	1–3	O	O	1–2	O–+	P and R

C = confluent; + = few; ++ = moderate number; +++ = innumerable; O = none detectable; R = regression; P = progression.

The lungs of some of the animals had quite extensive inflammatory reactions which were not typical of changes due to dust and vole bacillus and yet were often superimposed on lesions. The cause of this inflammation was not determined, and it is uncertain if it was a modified pseudo-tuberculosis.

The changes observed are summarized in Table II.

Lymph nodes.—We have often observed that the tracheobronchial lymph nodes appeared to reflect in a quantitative way the changes in the lungs. Nodes from all animals which received the dust plus vole bacillus and which were killed at the termination of the experiment were weighed and the results analysed statistically (Byers, 1959). The effect of Triton in the coal plus vole bacillus-injected animals was not significant ($p>0{\cdot}3$). The mean weight of the Triton-treated kaolin plus vole-injected animals (0·26 g.) was only about half that for the untreated kaolin plus vole-injected animals (0·51 g.), $p<0{\cdot}001$. The inference may be justified that in an infective pneumoconiosis the effect of Triton may depend on the nature of the dust. It is also possible that the effect of Triton is

related to the virulence of the organism, since it appears to have been more effective in this experiment than in our previous one with the photo-chromogenic mycobacterium (Byers and King, 1959).

SUMMARY

The surface active polyoxyethylene ether, Triton, did not appreciably alter the pathology produced in the lungs of guinea-pigs by the combined action of coal dust and *Myco. tuberculosis* (var. *muris*). With kaolin and mycotuberculosis it somewhat diminished the severity of the lesions. Tubercle bacilli survived longer in the lungs of untreated than in those of Triton-treated animals.

We should like to thank the Medical Research Council and the National Coal Board for financial assistance, and Dr. D. Weiss of the Sir William Dunn School of Pathology, Oxford, who kindly supplied us with cultures of the organism. One of us (P. D. B.) held the Allan Blair Memorial Fellowship of the Canadian Cancer Society.

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EXPLANATION OF PLATES

FIG. 1.—Guinea-pig lung 135 days after the intratracheal injection of 100 mg. of coal-mine dust, plus twice weekly subcutaneous injections of Triton 125 mg./kg.; no fibrosis. Silver impregnation ×13.

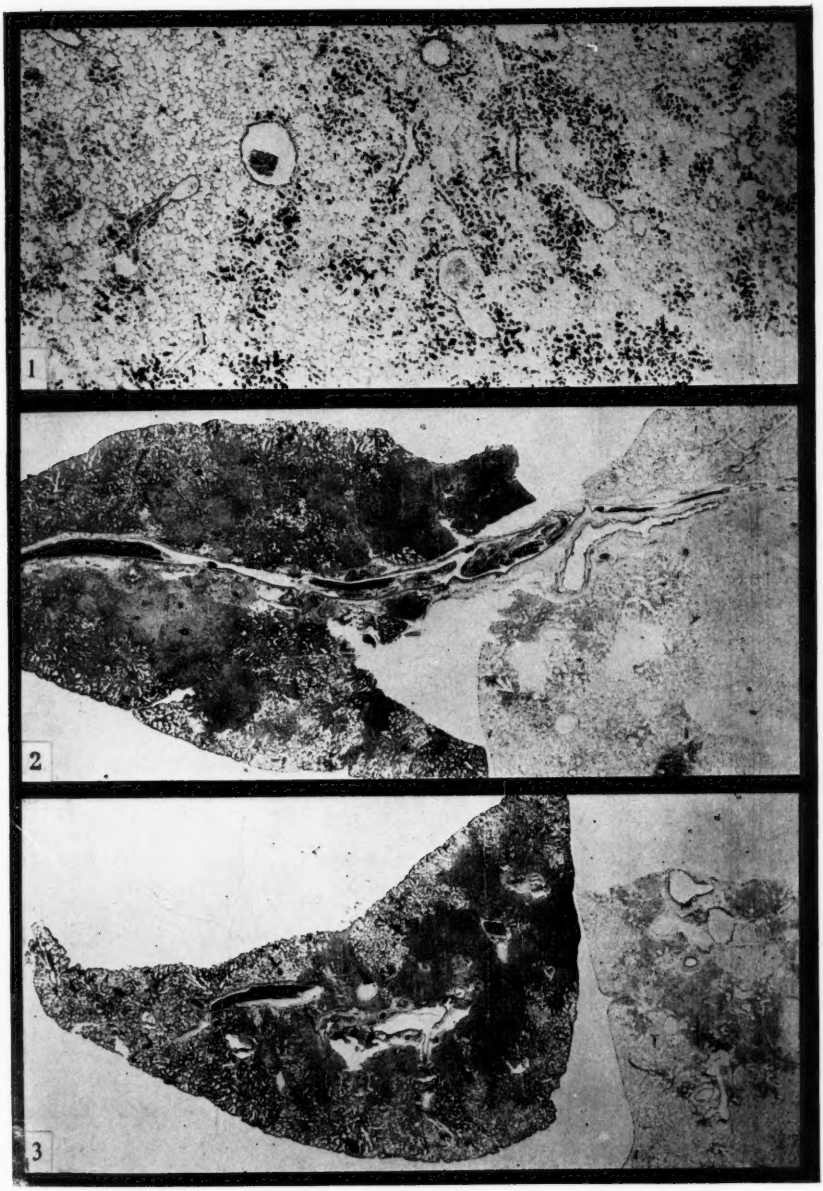
FIG. 2.—One hundred and thirty-five days after the injection of 100 mg. coal-mine dust plus 10^4 vole bacilli (see text) followed at 68 days by a second injection of 10^4 vole bacilli. Well-developed lesion with fairly abundant collagen; fibrosis Grade 2; necrosis in centre of lesion. Silver impregnation ×10.

FIG. 3.—One hundred and thirty-five days after 100 mg. coal-mine dust plus 10^4 vole bacilli (see text) followed at 68 days by a second injection of 10^4 vole bacilli, followed by twice weekly subcutaneous injections of Triton 125 mg./kg. Extensive lesion with fairly abundant collagen; no necrosis; fibrosis Grade 2. Silver impregnation ×10.

FIG. 4.—Guinea-pig lung 135 days after the intratracheal injection of 100 mg. of kaolin dust plus twice weekly subcutaneous injections of triton 125 mg./kg.; Grade 1 fibrosis. Silver impregnation ×10.

FIG. 5.—One hundred and thirty-five days after an initial injection of 100 mg. kaolin dust plus 10^4 vole bacilli (see text), followed at 68 days by a second injection of 10^4 vole bacilli. Extensive lesion in lower lobe with small areas of necrosis. Haematoxylin and eosin ×3.

FIG. 6.—One hundred and thirty-five days after an initial injection of 100 mg. kaolin dust plus 10^4 vole bacilli (see text), followed at 68 days by a second injection of 10^4 vole bacilli, followed by twice weekly subcutaneous injections of Triton 125 mg./kg. Extensive lesions in lower lobe; no necrosis. Haematoxylin and eosin ×3.



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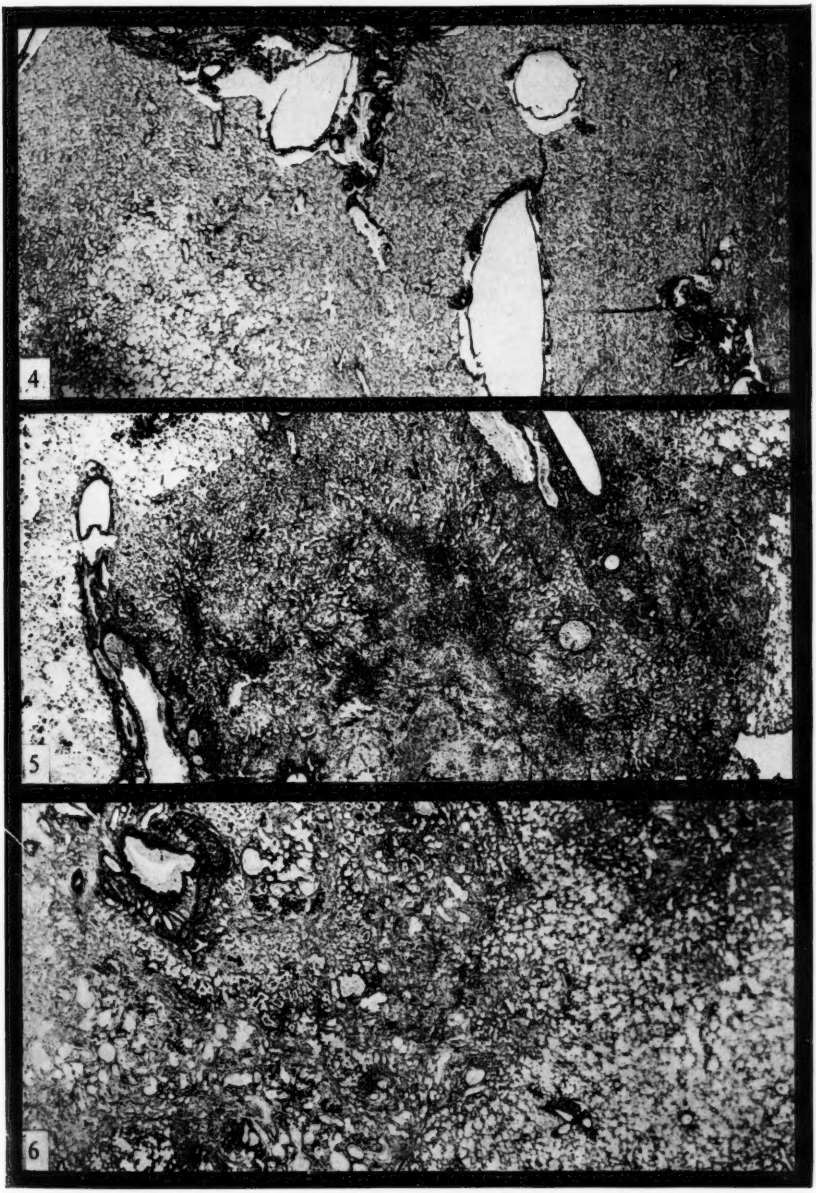

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HAEMORRHAGIC ACTIVITIES OF HABU SNAKE VENOM, AND THEIR RELATIONS TO LETHAL TOXICITY, PROTEOLYTIC ACTIVITIES AND OTHER PATHOLOGICAL ACTIVITIES

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THE most striking effects produced by the parenteral injection into animals of many snake venoms, especially of Crotalidae venoms, are local, consisting of haemorrhage, necrosis, and oedema (van Heyningen, 1954 ; Slotta, 1955). Mitsuhashi *et al.* (1959) reported that the pathological signs characteristic of Habu (*Trimeresurus flavoviridis*) bite are haemorrhage and necrosis, and that necrosis is severest in areas rich in muscular tissue.

Haemorrhage and necrosis are believed to be due, at least in part, to the action of proteolytic enzymes. Houssay (1930) and others (Kellaway, 1939 ; Zeller, 1948 ; Porges, 1953 ; van Heyningen, 1954 ; Kaiser and Michl, 1958 ; Maeno and Mitsuhashi, 1958) considered that proteolytic enzymes were mainly responsible for haemorrhage and necrosis at the site of the bites, while Slotta (1955) regarded them as being attributable mainly to the joint action of proteases and phosphatases.

The concept generally accepted that the effect of snake venom is manifest either locally or systemically does not hold true, because many venoms, including Habu venom, have both local and systemic elements. For elucidation of the pathological changes in animals caused by snake venom, not only local effects such as haemorrhage, necrosis and oedema but also systemic or lethal effects should be taken into consideration. It has been reported that the lethal toxicity of this venom was independent of such enzyme activities as phospholipase A, phosphodiesterase, 5′-nucleotidase and proteinase (with casein as substrate), which had been claimed to possess toxicological significance (Ohsaka, 1958 and 1960).

Here attention is concentrated on the following two points : firstly to elucidate the chemical nature of the principle responsible for haemorrhage or necrosis and secondly to show whether or not the principle responsible for the local effects also has the lethal toxicity or systemic effect. However, difficulties both in the quantitative estimation of haemorrhagic and necrotic activities and in the strict chemical separation of individual components have hindered solution of these problems.

Recently we have established a quantitative method for estimation of haemorrhagic activity—one of the most striking local actions of Habu venom (Kondo, Kondo, Ikezawa, Murata and Ohsaka, 1960). It consists of intracutaneous injections of venom into the depilated back skin of rabbits, a special device for accurate measurement of haemorrhagic areas and application of the parallel line assay method for the estimation. This has made it possible to isolate and charac-

terize the principle responsible for haemorrhage and to test the relation of haemorrhage to proteolytic activities and other pathological changes.

In the present work the venom of Habu (*Trimeresurus flavoviridis*) was fractionated on a large scale by zone electrophoresis employing starch as a supporting medium in an attempt to elucidate the relationships of haemorrhagic activity to lethal toxicity, proteolytic and other pathological activities.

MATERIALS AND METHODS

Snake venom.—The venom used was a dried and powdered pool (Batch No. 48) collected in Amami Oshima, a southern island of Japan, in 1948 from a species of Habu, *Trimeresurus flavoviridis* (Hallowell). The venom was reconstituted to the desired potency in physiological saline solution immediately before use.

Substrate.—Purified casein was purchased from Merck and Co., Inc., Rahway, N.J., U.S.A.

Experimental animals.—An inbred strain of white mice (general purpose colony, Yoken) of both sexes weighing 14–17 g. were used to determine lethal toxicity. Commercial adult white rabbits of both sexes weighing about 2·5 kg. were employed to determine the haemorrhagic activity.

Estimation of protein.—The protein content of the venom solution was estimated in terms of its ultra-violet absorption at 280 mμ in the 1 cm. cell of a Beckman DU spectrophotometer.

Estimation of proteolytic activity.—The proteolytic activity on casein was estimated by the modified method of Kunitz (1946), as previously described (Ohsaka, 1960). The reaction mixture contained 0·5 ml. of 4 per cent casein dissolved in 0·1 M disodium phosphate solution, 1·0 ml. of enzyme solution and 0·5 ml. of 0·2 M phosphate buffer (pH 7·5). After 10 min. incubation at 35°, the reaction was stopped by the addition of 2·0 ml. of 0·4 M trichloroacetic acid. The extent of digestion was evaluated by determination of the absorption at 280 mμ of the trichloroacetic acid filtrate.

Lethal toxicity.—Lethal toxicities were assayed by intravenous injections of mice with 4–5 doses of each fraction increasing by 1·4-fold steps in volumes of 0·2–0·5 ml. Five animals were injected with each dose. The range of dilutions inoculated covered from 0 to 100 per cent mortality. All deaths during the 4 days following injection were ascribed to venom toxicity, although most of the deaths occurred before 24 hr. The LD_{50} was calculated by the Reed-Muench method. The standard error of the LD_{50} was calculated according to Pizzi (1950), the fiducial limits of which are 30 per cent for a probability of 0·05. The LD_{50} of the crude venom used (Batch No. 48) was 61 μg. $\pm$ 3 μg. as estimated by a statistical analysis of the results from titrations repeated 30 times, carried out in the above-described manner.

Assay of haemorrhagic activity.—The haemorrhagic activity was assayed by the method previously described (Kondo *et al.*, 1960). Series of 4–5 doses of 0.1 ml. of each fraction graded with 3-fold intervals were injected intracutaneously into the depilated back skin of 2–3 rabbits weighing about 2·5 kg. The rabbits were killed by chloroform inhalation 24 hr. later and the skins were removed immediately. The skin was spread and fixed on a glass plate so as not to distort the original size. The cross-diameters of each haemorrhagic spot were measured from the visceral side of the skin through the glass plate and the mean of the 2 diameters was taken as an indicator of the intensity of the response. The results were analysed statistically. As previously described (Kondo *et al.*, 1960) the minimum haemorrhagic dose (MHD) of venom was defined as the least quantity of venom, which caused a haemorrhagic reaction 10 mm. in diameter 24 hr. after intracutaneous injection under the above conditions. The MHD of the crude venom used (Batch No. 48) was approximately 0·20 μg., its fiducial limits being $\pm$25 per cent for a probability of 0·05.

Histological examination.—In each experiment 0·1 ml. series of several doses of several fractions increasing by 3-fold steps were injected intracutaneously into the depilated back skin of one rabbit. The rabbit was killed 24 hr. after injection by chloroform inhalation and blocks of tissue were taken from the skin. These were fixed in Orth's solution and embedded in paraffin. Sections were cut at a thickness of about 3 μ and stained with Carazzi's haematoxylin and eosin.

Zone electrophoresis.—Zone electrophoresis in a column using starch as the supporting medium was carried out in a vertical-type apparatus with a few of the modifications of Flodin and Porath (1954). Commercial potato starch was thoroughly washed and treated with buffer (Ohsaka, 1958). Runs were made in borax-NaOH buffer of pH 9·20 and 0·1 ionic strength in a cold room with an ambient temperature of 2–4°. The column consisted of a plastic tube 120 cm. long and 3·6 cm. in diameter with an outer cooling jacket (diameter 5 cm.) along its whole length. Water from a thermostatic bath circulating through the jacket might have substituted for the cold room treatment. The starch packing was supported by 2 sheets of filter paper (Toyo Roshi No. 26, for the filtration of oils) which were fixed at the base of the column by a close-packed screwed plug. The total length of the packed column was 114·5 cm., the cross-sectional area 10·2 cm.^{2} and the ratio of starch volume/mobile liquid volume 2·0. The hold-up volume was 1164 ml. The sample of crude venom amounting to about 2·0 g. in 20 ml. of physiological saline, was introduced in the starch stratum of about 5·9 cm. in thickness at the height of 87 cm. The anode was connected to the bottom of the packed column through the tube filled up with buffer. A current of 20 mA with a potential drop of about 380 volts was passed for about 75 hr. At the end of the electrophoresis the starch column was eluted with the same buffer at a flow rate of 31·8 ml./hr. and 10·6 ml. fractions were collected by a fraction collector. The amount of protein, the proteolytic activity, the lethal toxicity and the haemorrhagic activity in each fraction was determined.

RESULTS

Evidence for the presence of two haemorrhagic activities and their relations to lethal toxicity

The elution diagram of Habu venom subjected to zone electrophoresis in a packed starch column is presented in Fig. 1 and 2. The electrophoretic conditions are given in Fig. 1 and the arrow represents the place where crude venom was applied. The pattern was reproduced in a duplicate experiment. The protein recovery was 97 per cent. The distribution of haemorrhagic activity, lethal toxicity and proteolytic activity on casein in each fraction were surveyed with the results shown in Fig. 1 and 2. In Table I are recorded the haemorrhagic activity of each fraction and its fiducial limits. The total recovery was 71 per cent (57–89 per cent) for haemorrhagic activity and 71 per cent for lethal toxicity.

It is evident in Fig. 1 that the venom contained at least 2 haemorrhagic fractions. For the sake of convenience, the larger peak was designated HR1 and the smaller one HR2. Both peaks were located apart from each other on the starch column under the conditions used here : HR1 peak was found in fraction No. 22 and HR2 peak in fraction No. 14. The recovery was 46 per cent for HR1 and 25 per cent for HR2. Calculating from Table I the specific activity expressed as its ratio to that of the crude venom was 1·4 for HR1 and 3·2 for HR2 peak.

The difference between HR1 and HR2 in the electrophoretic mobility was further confirmed by subsequent electrophoresis when the 2 fractions were introduced separately into parallel troughs of the same size (Ohsaka *et al.*, 1960).

Fig. 1 also demonstrates that HR2 can be separated from the main lethal toxicity peak (Fractions No. 17–26) while it is overlapping the minor part of lethal toxicity (Fractions No. 11–16). Therefore, it is evident that HR2 is unrelated to, at least the main part of, lethal toxicity.

As for HR1, Fig. 1 proves that the peak is overlapping the main lethal toxicity peak.

Relations of haemorrhagic activity to proteolytic activity on casein

Fig. 2 shows that this venom contained at least 5 electrophoretic components

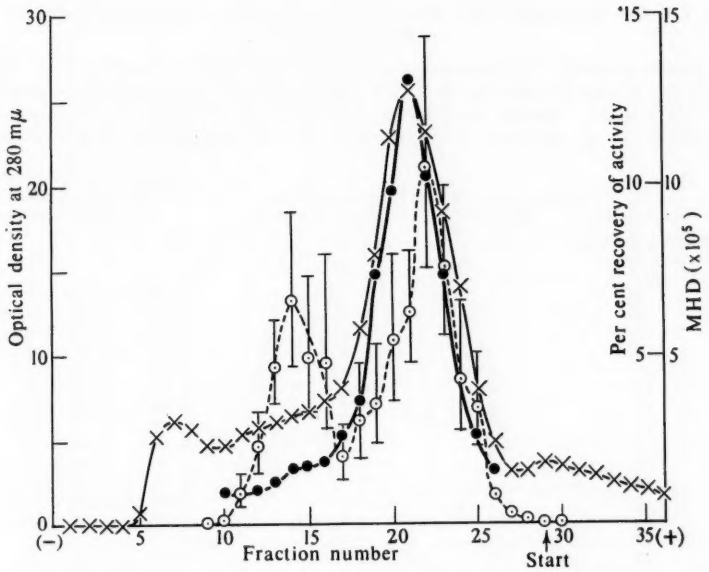

FIG. 1.—Distribution of protein (×————×), haemorrhagic activity (○- - - - -○) and lethal toxicity (●————●) in eluate from column after zone electrophoresis. A quantity of 2·0 g. of Habu venom was subjected to electrophoresis in a column of 3·6 × 114·5 cm. packed with starch. The run was made in borax-NaOH buffer of pH 9·20 and 0·1 ionic strength in a cold room (2–4°). A current of 20 mA with a potential difference of 380 volts was passed for about 75 hr. 10·6 ml. fractions were collected.

having proteolytic activity on casein (Ohsaka, 1960). The total recovery of proteolytic activity was 100 per cent with casein. The possibility that the 5 electrophoretic components represent distinct enzymes was investigated and reported separately (Ohsaka, 1960); where it was shown that these components included at least 3 distinct enzymes.

It is evident that both HR1 and HR2 were separable from the peak of proteolytic activity on casein as found in fraction No. 17. Distribution of proteolytic activity on " azocoll " lacked a peak in fraction No. 22 (Ohsaka, 1960). Therefore, HR1 must be independent of proteolytic activity on " azocoll " (Oakley, Warrack and van Heyningen, 1946). As can also be seen in Fig. 2, however, HR1 and HR2 peaks coincided with those of proteolytic activity on casein as were found in fraction No. 22 and fraction No. 14, respectively, suggesting the possibility that haemorrhagic activity might be correlated with the proteolytic activity on casein.

Relations of muscle degenerating effect to haemorrhagic activity and proteolytic activity on casein

Fig. 3 and 4 show the typical histopathological changes produced in the back skin of rabbits 24 hr. after intracutaneous injection of various amounts of crude Habu venom.

TABLE I.—*Haemorrhagic Activity of Each Fraction Separated by Starch Column Electrophoresis*

Electrophoretic run is the same as described in Fig. 1. Fraction No. in this Table is also the same as in Fig. 1. Haemorrhagic activity is expressed by MHD units. Specific activity of haemorrhagic activity is expressed by MHD per μg. protein. Fiducial limits of each estimation are also shown.

Fraction No.	Number of spots observed	Specific activity (MHD/μg protein)	Activity recovered and its fiducial limits (10^5 MHD/fraction)	
Starting material (crude venom 2·0 g.)	30	5·00	100	(80–125)
9	4	0·00735	0·00213	(0·00127–0·00358)
10	4	0·358	0·105	(0·0625–0·176)
11	4	2·50	0·877	(0·522–1·48)
12	7	6·25	2·25	(1·51–3·32)
13	16	11·8	4·61	(3·54–6·00)
14	9	16·1	6·54	(4·64–9·22)
15	8	11·5	4·89	(3·26–7·33)
16	4	9·90	4·73	(2·81–7·94)
17	8	3.73	1·96	(1·30–2·94)
18	5	4·00	3·01	(1·90–4·76)
19	8	3·33	3·51	(2·34–5·27)
20	7	3·57	5·38	(3·64–7·96)
21	17	3·70	6·20	(4·77–8·06)
22	10	6·90	10·5	(7·58–14·4)
23	11	6·25	7·55	(5·56–10·0)
24	6	4·61	4·22	(2·72–6·53)
25	7	6·54	3·38	(2·28–5·00)
26	18	2·57	0·815	(0·632–1·05)
27	3	1·37	0·275	(0·154–0·490)
28	4	0·524	0·105	(0·0625–0·176)
29	2	0·125	0·0304	(0·0145–0·0638)
30	2	0·0752	0·0180	(0·00857–0·0378)
		Sum	71·0	(56·8–88·7)

Fig. 3 shows a typical picture observed when 30 μg. of crude venom was injected. Haemorrhage and infiltration by polymorphonuclear leucocytes were usually observed in the corium. Accompanying muscular degeneration in the muscular layer was observed and in most instances, infiltration by polymorphonuclear leucocytes. Haemorrhage appeared in the muscular layer to an extent which depended upon the amount of venom injected, as exemplified in Fig. 4 where a larger amount of venom was inoculated. In short, haemorrhage in the corium and waxy degeneration in the muscular layer were usually observed. Muscular degeneration proceeded to necrosis or death of the muscle fibres in its severer forms (Fig. 4). These appeared to be the main characteristics of histopathological changes caused by Habu venom.

In order to examine the relationship of the muscle degeneration effect, which leads to necrosis in its severer forms, to haemorrhagic activity and proteolytic activity on casein, histopathological changes evoked in the rabbit skin by intracutaneous injection of several fractions were studied comparatively. These results are summarized in Table II.

It is apparent that an amount as small as 1–0·1 μg. of crude venom could cause haemorrhage (Fig. 5) whereas more than 3–10 μg. was needed to cause muscular degeneration (Fig. 6). The specific haemorrhagic activity in fractions

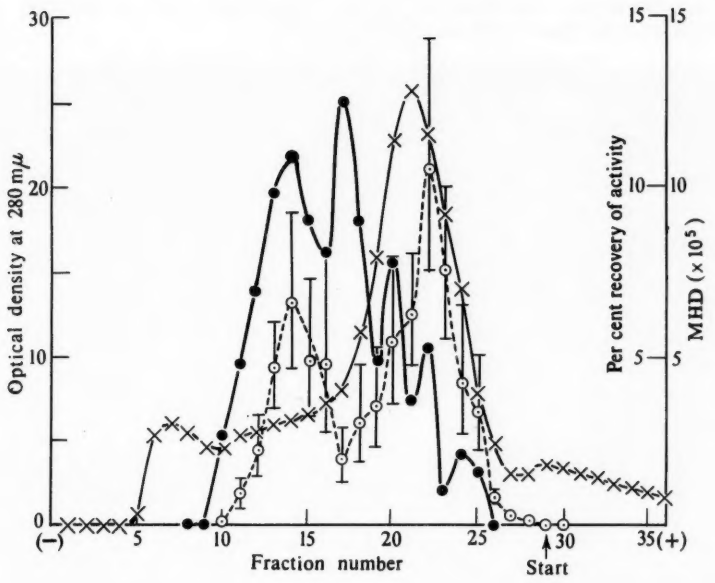

FIG. 2.—Distribution of protein (×————×), haemorrhagic activity (○- - - -○) and proteolytic activity on casein (●————●) in eluate from column in the same electrophoretic run as in Fig. 1.

13 and 14 were 2·4 times and 3·2 times as large as in crude venom respectively. Nevertheless, they showed rather weaker muscle degenerating effects than crude venom (Fig. 7 and 8). Therefore, it is suggested that the muscle degeneration effect did not run parallel to haemorrhagic activity.

Table II also shows that the specific proteolytic activity on casein in fraction 13 was 5-fold and in fraction 14 5·4-fold larger than in crude venom. Yet, they showed rather weaker muscle degeneration effects (Fig. 7 and 8). On the other hand, despite the absence of detectable proteolytic activity on casein, fraction 26 showed very much the same muscle degeneration effect as crude venom (Fig. 9). This observation strongly suggested the absence of a parallel between the muscle degeneration effect and the proteolytic activity on casein.

DISCUSSION

The new quantitative method for estimating haemorrhagic activity proposed by us (Kondo *et al.*, 1960), coupled with electrophoretic fractionation on a large scale in a packed starch column, of Habu snake venom made it possible to study the principle responsible for haemorrhage.

The data presented (Fig. 1) indicate that at least 2 haemorrhagic fractions, different in electrophoretic mobility, were present in this venom (HR1 and HR2). Difference in mobility between HR1 and HR2 was confirmed by repeated electrophoresis (Ohsaka *et al.*, 1960).

TABLE II.—*Summary of Histopathological Changes Evoked in the Rabbit Skin by Intracutaneous Injection of Crude Venom as well as Several Fractions Separated by Starch Column Electrophoresis*

Electrophoretic run is the same as described in Fig. 1.

Amount of injection (μg. protein)	Histopathological changes: Crude venom (Pr = 1, Hr = 1) Exp. No. 1 H	Crude venom (Pr = 1, Hr = 1) Exp. No. 1 MD	Crude venom (Pr = 1, Hr = 1) Exp. No. 2 H	Crude venom (Pr = 1, Hr = 1) Exp. No. 2 MD	Fraction No. 13 (Pr = 5·0, Hr = 2·4) Exp. No. 1 H	Fraction No. 13 (Pr = 5·0, Hr = 2·4) Exp. No. 1 MD	Fraction No. 14 (Pr = 5·4, Hr = 3·2) Exp. No. 2 H	Fraction No. 14 (Pr = 5·4, Hr = 3·2) Exp. No. 2 MD	Fraction No. 26 (Pr = 0, Hr = 0·5) Exp. No. 1 H	Fraction No. 26 (Pr = 0, Hr = 0·5) Exp. No. 1 MD
300 μg.	..	..	+	+	+	+	+	+	+	+
100	+	+	+	+	+	±	+	+	+	+
30	+	+	+	+	+	±	+	+	+	+
10	+	+	+	+	+	−	+	±	+	±
3	+	−	+	+	+	−	+	−	+	−
1	+	−	+	−	+	−	+	−	..	..
0·3	±	−	+	−	+	−	+	−	..	..
0·1	−	−	+	±	−	−	+	−	..	..
0·03	−	−	−	−	−	−	+	−	..	..

Pr : Specific activity of proteolytic activity on casein.
Hr : Specific activity of haemorrhagic activity.
H : Haemorrhage in histopathological picture.
MD : Muscular degeneration or necrosis of muscle fibres in histopathological picture.
+ : Presence of waxy degeneration or necrosis of muscle fibres ,or presence of haemorrhage.
± : Doubtful degeneration of a few muscle fibres or doubtful haemorrhage in the whole section of the examined tissue of the skin.
− : Without degeneration of muscle fibres or without haemorrhage.

EXPLANATION OF PLATES

Figs. 3–9 are all of the same magnification (×85).

FIG. 3.—Section of the rabbit skin inoculated with venom (1) : From Expt. 2 (crude—30 μg.). Muscle fibres show waxy degeneration (MD) with infiltration of polymorphonuclear leucocytes (L) around them. Haemorrhage (H) is seen between muscle fibres. (H and E.) ×85.

FIG. 4.—Section of the rabbit skin inoculated with venom (2) : From Expt. 3 (crude—300 μg.). Almost all muscle fibres show the necrosis and dissolution. Diffuse infiltration of polymorphonuclear leucocytes (L) and remarkable haemorrhage (H) are found.

FIG. 5.—Section of the rabbit skin inoculated with venom (3) : From Expt. 2 (crude—0·1 μg.). Haemorrhage (H) is found in the corium and remarkable hyperaemia (HY) in the profound layer of the corium above the muscular layer. No muscle fibres show degeneration.

FIG. 6.—Section of the rabbit skin inoculated with venom (4) : From Expt. 2 (crude—3 μg.). A few muscle fibres show waxy degeneration (MD) with infiltration of polymorphonuclear leucocytes (L) in and around them. Haemorrhage (H) is seen between muscle fibres.

FIG. 7.—Section of the rabbit skin inoculated with venom (5) : From Expt. 1 (Fraction No. 13—100 μg.).
No muscle fibres show degeneration. But remarkable haemorrhage (H) between muscle fibres is found.

FIG. 8.—Section of the rabbit skin inoculated with venom (6) : From Expt. 2 (Fraction No. 14—10 μg.).
No muscle fibres show degeneration. But haemorrhage (H) between them is found.

FIG. 9.—Section of the rabbit skin inoculated with venom (7) : From Expt. 1 (Fraction No. 26—30 μg.).
A few muscle fibres show waxy degeneration (MD) with infiltration of polymorphonuclear leucocytes (L) around them. Haemorrhage (H) is seen between muscle fibres.

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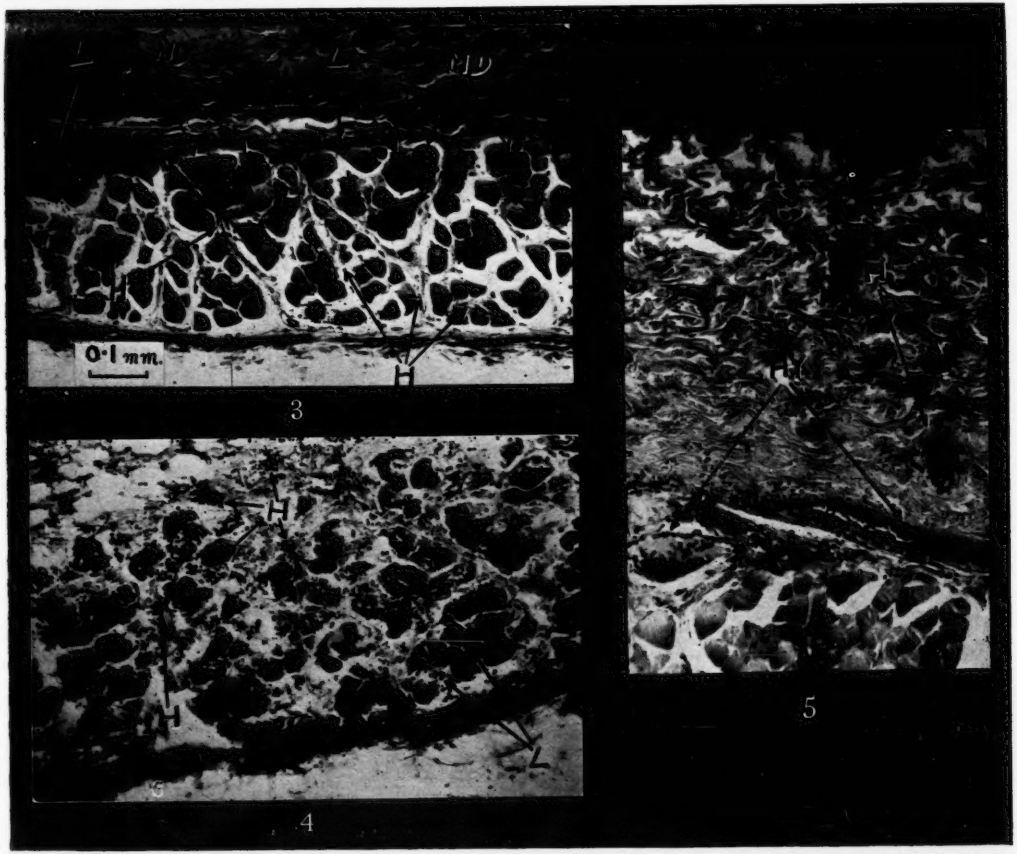
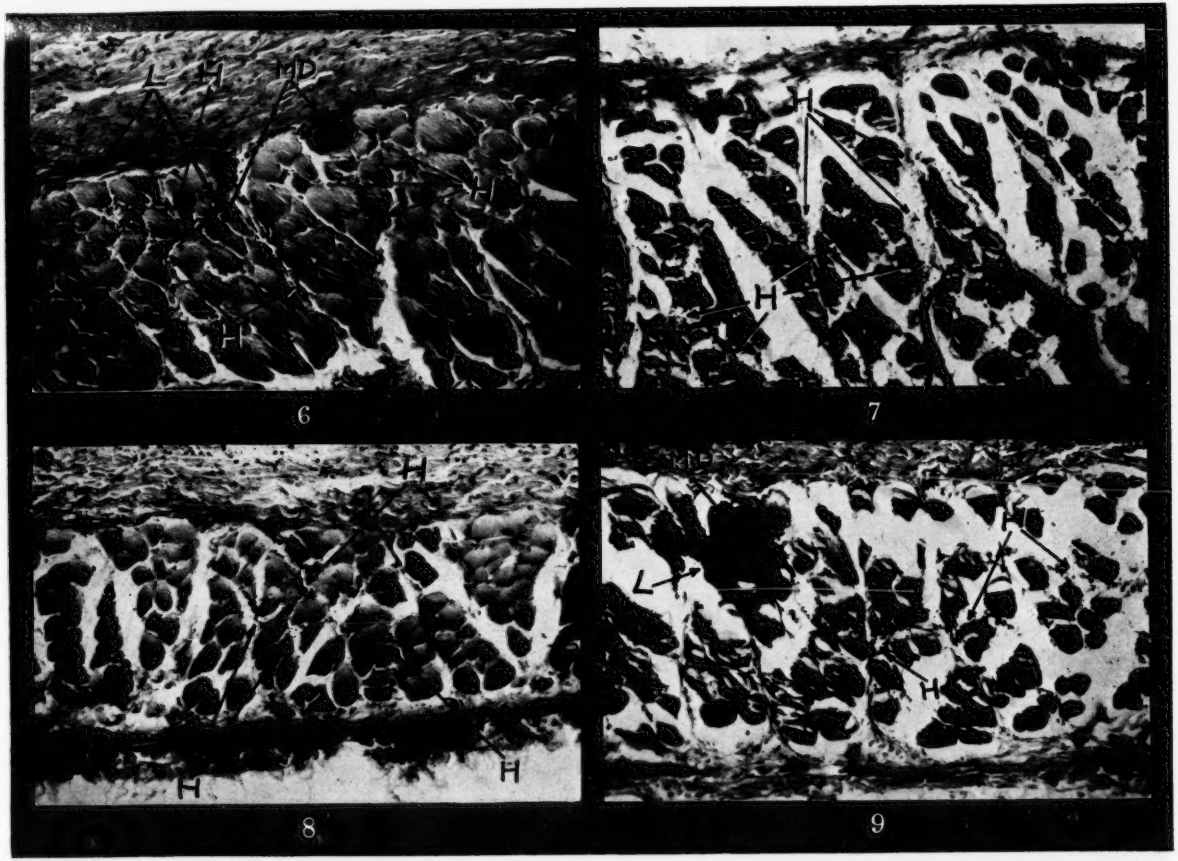


Ohsaka, Ikezawa, H. Kondo, S. Kondo and Uchida.


L
H
MD
H
H
6
H
H
7
H
H
H
8
L
H
H
9

It is an interesting problem whether or not the principle responsible for haemorrhage can also have the lethal toxicity. Fig. 1 shows that HR2 can be separated from the major part of lethal toxicity (fractions 17–26) while overlapping the minor part of lethal toxicity (fractions 11–16). Therefore, it is evident that HR2 has no relation with, at least the main part of, lethal toxicity. On the other hand, the HR1 peak is overlapping the main lethal toxicity peak (Fig. 1). However, a small difference in mobility between the HR1 and the main lethal toxicity peaks (Fig. 1) led us to entertain an idea that both might be separate entitites. Further work is required to establish the relationship of haemorrhagic activity to lethal toxicity.

Houssay (1930) and others (Kellaway, 1939 ; Zeller, 1948 ; Porges, 1953 ; van Heyningen, 1954 ; Slotta, 1955 ; Kaiser and Michl, 1958) considered that haemorrhage is due, at least in part, to the action of proteolytic enzymes. Recently Maeno and Mitsuhashi (1958) proposed that a proteinase (substrate casein) partially purified from Habu venom was the only factor responsible for haemorrhagic necrosis. Their claim, however, was not based on any quantitative evidence. To elucidate the relationship of haemorrhagic activity to proteolytic activity on casein, experiments were carried out on a quantitative basis. The results testified that both HR1 and HR2 could be regarded as being different from the proteolytic activity peak with casein as found in fraction 17 (Fig. 2). HR1 can be separated from the proteolytic activity on " azocoll ". However, the peaks HR1 and HR2 coincided with those (fractions 22 and 14) of proteolytic activity on casein (Fig. 2). It appears that a correlation exists between haemorrhagic activity and proteolytic activity on casein. However, it seemed that repeated electrophoresis could further fractionate HR2 into 2 components, one of which was separable from the proteolytic activity (Ohsaka *et al.*, 1960). Further studies are necessary to examine the possible correlation between haemorrhagic and proteolytic activities.

Histological observations indicated that the easily discernible pathological changes produced in the back skin of rabbits by intracutaneous injection of crude Habu venom were haemorrhage in the corium and muscular degeneration in the muscular layer. Muscular degeneration proceeded to necrosis or death of the muscle fibre in its severer forms.

It is of great interest whether or not the same principle is responsible for necrosis and haemorrhage. However, little is known concerning this point. Kaiser and Michl (1958) considered that venom causes severe necrosis at the site of the bite as a result of its proteolytic action on tissue and furthermore proteolytic enzymes, when very active, cause dissolution of small blood vessels, resulting in haemorrhage. In other words, they insinuated that one and the same principle was responsible for necrosis and haemorrhage. However, histopathological observations presented here indicated that the muscle degeneration effect, whose severer forms would be manifested as necrosis, did not run parallel to haemorrhagic activity (Table II).

Necrosis is also believed to be due, at least in part, to the action of proteolytic enzymes (Houssay, 1930 ; Kellaway, 1939 ; Zeller, 1948 ; Porges, 1953 ; van Heyningen, 1954 ; Slotta, 1955 ; Kaiser and Michl, 1958 ; Maeno and Mitsuhashi, 1958). However, Table II also showed that the muscle degeneration effect did not run parallel to proteolytic activity on casein.

Further purification of haemorrhagic and lethal principles are being continued.

SUMMARY

The venom of *Trimeresurus flavoviridis* (Habu), a crotalid, was fractionated on a large scale by zone electrophoresis in a packed starch column. Haemorrhagic activity, lethal toxicity and proteolytic activity in each fraction were determined quantitatively.

It was found that at least 2 haemorrhagic principles, designated as HR1 and HR2, were involved in this venom. HR2 could be separated from the main part of lethal toxicity. Both HR1 and HR2 were associated with proteolytic activity on casein.

Histopathological observations indicated that the muscle degeneration effect was related neither to haemorrhagic activity nor to proteolytic activity on casein, as far as herein tested.

The authors are deeply indebted to Dr. D. Mizuno, Chief of Department of Biochemistry, to Dr. R. Murata, Chief of Department of Serology and to Dr. Y. Egashira, Chief of Department of Pathology, of the National Institute of Health, Tokyo, for their helpful advice and keen interest in this work.

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VASCULAR PERMEABILITY CHANGES IN INFLAMMATION: THE ROLE OF ENDOGENOUS PERMEABILITY FACTORS IN MILD THERMAL INJURY

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IN the inflammatory response to injury, increased capillary permeability is an early and important vascular event. Research into the mediation of the permeability changes in general concerns endogenous substances with high permeability-increasing potency, on the grounds that they are likely to be the natural mediators in inflammation, particularly if they are widely and generously distributed in mammalian tissues and are readily activated.

Various endogenous permeability factors satisfying these criteria have been identified; and, in most cases, demonstrated in inflammatory exudates. However, their presence in exudates is insufficient to establish their relevance as the natural mediators of the appropriate permeability changes. This paper reports a more direct investigation of the role of endogenous permeability factors in experimental thermal injury.

Established candidates for this role can be divided into two groups; on one hand, the amines, histamine (Lewis, 1927) and 5-hydroxytryptamine (Rowley and Benditt, 1956), and their liberators (see Paton, 1951); and on the other, proteases and the products of proteolysis. The second group includes the protease, plasmin (Ungar, 1952); and globulin permeability factor, which is probably a protease (Mackay, Miles, Schachter and Wilhelm, 1953); as well as polypeptides like leukotaxine (Menkin, 1938), bradykinin (Rocha é Silva, Beraldo and Rosenfeld, 1949) and kallidin (Werle, Gotze and Keppler, 1937).

Permeability changes are readily investigated in the skin of animals with a vital dye like pontamine blue circulating in their blood, increased permeability to large molecules (see Rawson, 1942–43; Miles and Wilhelm, 1960*a*) being indicated by local exudation of blue dye at a treated site.

These permeability factors when injected intracutaneously increase permeability in general for only about 15 min. On the other hand, the increased permeability in inflammation, besides occurring within minutes, often persists for hours or days. The short-lived effect of the isolated factors may raise doubt about their natural role in injury; but it also emphasizes that a satisfactory assessment of their role requires knowledge of the precise time-course of the permeability changes induced by a standard stimulus. This argument applies whatever the strategy of identifying the natural mediators in injury—whether by isolating the permeability factors from the affected site, or by demonstrating suppression of the permeability response in animals either treated with specific inhibitors or depleted of the factors.

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The difficulties of attempted isolation and the shortcomings of depletion experiments are discussed in detail elsewhere (Miles and Wilhelm, 1960*a*). Briefly, the attempted isolation from skin of the natural mediators is complicated by the ready release of histamine in practically any tissue manipulation, and by the normal presence in the tissue fluid of skin of the globulin permeability pro-factor, which is readily activated by *in vitro* dilution or chemical fractionation (Miles and Wilhelm, 1955, 1958; Wilhelm, Miles and Mackay, 1955). Histamine depletion in rats induces a non-specific decrease in the animals' reactivity to various permeability factors like histamine, a histamine-liberator and a non-liberator (Wilhelm, Mill, Sparrow, Mackay and Miles, 1958); and attempts to deplete animals of globulin factor are not feasible (Wilhelm, Mill and Miles, 1957). Accordingly we have at this stage mainly studied the effects of various inhibitors on the permeability changes in experimental injury.

Thermal injury was induced in guinea-pigs, rats and rabbits by the minimal stimulus that consistently elicited a permeability response. A minimal stimulus has two advantages (Wilhelm and Mason, 1958)—it reduces the possibility of complicating the pathological picture by non-specific breakdown products; and, if it is not painful it permits the work to be done in unanaesthetized animals, thereby avoiding the depression of the permeability response induced by anaesthesia (Miles and Miles, 1952).

MATERIALS AND METHODS

Animals.—Albino guinea-pigs (600–800 g.) and rats (260–320 g.) and non-moulting rabbits (2·5–3·0 kg.), either albino of mixed laboratory stock or New Zealand red, were used. The skin of the back of the trunk, from the base of the ears in front to the lumbo-sacral junction behind, and laterally to the mid-axillary line, was closely clipped, depilated with grey barium sulphide paste (Miles and Miles, 1952), thoroughly washed with tepid water and dried. To avoid " burning " rabbits' skin, the depilatory paste must be thin in consistency and applied for a minimum period. Rats' skin, 18 hr. after depilation, is scaly and erythematous; and in experiments lasting more than 12 hr., was satisfactorily depilated with a Philishave Dry Shaver (Philips Electrical Ltd., London).

Guinea-pigs were given intravenous pontamine blue, 36 mg./animal, and rabbits 60 mg./kg., as a 5 per cent solution in 0·425 per cent saline; rats were given 60 mg./kg. as a 2·5 per cent solution in 0·6 per cent saline. Such animals are referred to as " blued ".

Further technical details of testing permeability factors are given by Miles and Wilhelm (1955), and Wilhelm *et al.* (1958).

Unless stated otherwise, the animals were unanaesthetized throughout the experiments.

Thermal injury.—A polished copper disc, 9 mm. diam., closing one end of a copper tube and maintained by circulating water at a stated temperature ($\pm 0{\cdot}25°$), was applied to the depilated skin of guinea-pigs, rats and rabbits. The disc temperature was assumed to be that of the immediately adjacent water, recorded by a thermometer inserted through a rubber bung closing the other end of the tube. The apparatus is a modification of that used by Sevitt (1949).

Guided by the maximal thermal stimulus that we ourselves could tolerate on the skin on the inner aspect of the wrist, we determined the minimal painless stimulus that consistently elicited a permeability response on the back of the trunk of each species. The maximum heating applied to unanaesthetized animals—54° for 20 sec. to guinea-pigs and rats, and 56° for 20 sec. to rabbits—was comfortably tolerated in each case by animals lightly held by hand. The heated skin neither blisters nor becomes necrotic. For the application of stronger stimuli, the animals were anaesthetized with open ether.

Measurement of permeability effects.—The permeability effects of graded doses of preparations injected intracutaneously in blued guinea-pigs (Miles and Miles, 1952; Miles and Wilhelm, 1955), rats and rabbits (Wilhelm *et al.*, 1958) can be precisely estimated from the linear dosage-response lines obtained by plotting lesion-diam. against log. dose. In

such assays, colour-intensity is also roughly proportional to log. dose, but only lesion-diam. is usually plotted. The heated metal disc used in the present work to produce thermal injury induced lesions of constant diameter, and of necessity we have used colour-intensity as the index of permeability change. This procedure is justifiable because (*a*) in intracutaneous assays, colour-intensity is also roughly proportional to log. dose of a permeability factor (PF), and can therefore be used as an index of the amount of permeability change ; and (*b*) though the intensity is not a simple function of the amount of intracutaneous dye (Fig. 1), the relation of the two is empirically simple enough to use as a measure of large changes in permeability.

The intensity of colour was graded as follows : *trace*, $\pm$, $\pm$, $\pm$, $+$, $+\pm$, $++$ up to $++++$; *trace* being the faintest recognizable colour, and $++++$ a deep royal blue. In each experiment, a selected lesion was allotted colour-intensity $++$ and used as a standard. The inevitable slight differences in day-to-day grading were largely offset in each experiment by inducing multiple lesions in each of 3–4 guinea-pigs or rats, or multiple lesions duplicated in each of 2 rabbits ; many results are the means of several experiments. To determine the correlation in guinea-pigs between colour-intensity on our arbitrary scale and local concentration of dye, we made intracutaneous injections of graded concentrations of pontamine blue in guinea-pig serum. An increase in colour-intensity at the injected sites from *trace* to $\pm$ corresponded to a 10-fold increase in dye concentration ; and $\pm$ to $+++$ to a 20-fold increase in concentration (Fig. 1).

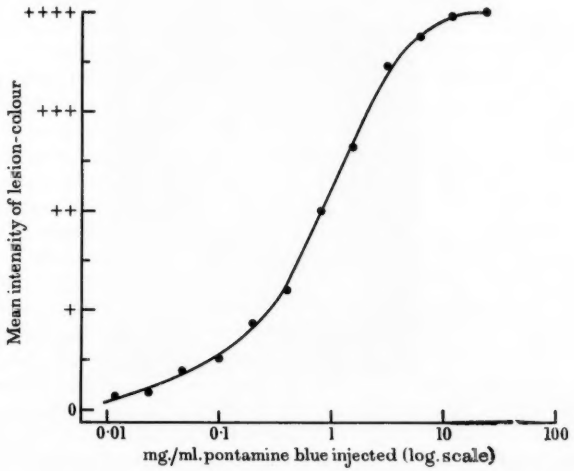

FIG. 1.—The relation between intensity of lesion-colour and the content of pontamine blue in guinea-pig skin injected with 0·05 ml. volumes of graded concentrations of dye.

Preparations for intracutaneous injection in guinea-pigs and rabbits were made up in 0·85 per cent saline ; for injection in rats, in Locke's solution (Wilhelm *et al.*, 1958).

Drugs.—Histamine was used as the acid phosphate (British Drug Houses) and 5-hydroxytryptamine (5-HT) as the creatinine sulphate (Upjohn Co., Kalamazoo, U.S.A.). The antihistamines chlorprophenpyridamine and mepyramine each were used as maleate, thenalidine as tartrate and the remainder (see Table I) as hydrochloride. The weights of histamine, 5-HT and antihistamines are cited as base.

Globulin permeability factor (globulin PF ; serum fraction G2/1R) was prepared from guinea-pig, rat and rabbit sera according to Mackay (1955) and Wilhelm *et al.* (1957).

Glycomucoid trypsin inhibitor was prepared by our former colleague, Dr. P. J. Mill, according to Lineweaver and Murray (1947).

Assay of histamine and 5-HT.—Preparations were assayed for histamine content on the

atropinized guinea-pig's ileum suspended in oxygenated Mg-free Tyrode solution in a 10 ml. bath at 34°; and for 5-HT content on the atropinized rat uterus in de Jalon's solution at 29°.

RESULTS

Thermal Injury in Guinea-pigs

At skin sites heated at 54°, the permeability response consists of two distinct phases: *immediate*—maximally induced by 5 sec. heating, lasting less than 10 min. and almost certainly mediated by histamine; and *delayed*—beginning about 1 hr. after 20 sec. heating, maximal in 3–4 hr., and apparently not induced by histamine or dependent on the preceding immediate response to histamine (Wilhelm and Mason, 1958).

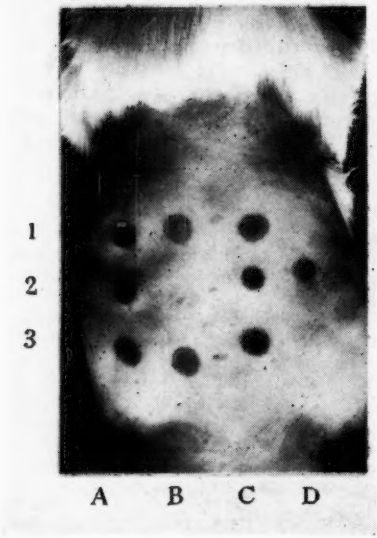

FIG. 2.—The immediate permeability response to 5 sec. heating at 54° in guinea-pig skin, and its suppression by systemic triprolidine.
A1 and 3, C1 and 3. Full response.
A2, C2. Full response in sites previously injected with saline (see text).
B1 and 3, D2. Response in sites heated after 10 μg. triprolidine/kg. intravenously.
B2, D1 and 3. Response in sites heated after a second intravenous injection of tripolidine, making a total dose of 100 μg./kg.
D3, B2, B1 and A3 exemplify intensities of blueing graded respectively as *trace*, ±, +, +++.

The immediate response

In blued guinea-pigs, skin sites heated at 54° for 5 sec. display only a monophasic response. The sites are at first slightly blanched, but become erythematous in 30–40 sec. and begin to blue after a further 5–10 sec. The blue colour rapidly increases in intensity to a maximum ++ to +++ in 5 min. (Fig. 2) and, in sec-

tions through the mature lesion perpendicular to the skin, is confined macroscopically to the upper two-thirds of the dermis. The normal low level of permeability of the capillaries in the heated site is restored in 10 min., as shown by the technique of Miles and Miles (1952), in which intravenous dye is given to animals bearing lesions of different ages.

The decline in permeability after 10 min. is not caused by vascular obstruction. Skin sites near the posterior axillary line were heated at 54° for 5 sec. and their vascular bed outlined by injecting a carbon suspension into the corresponding axillary artery in the anaesthetized animal (Burke and Miles, 1958). In heated sites 1½–20 min. old, the vascular bed filled normally.

The erythema, in unblued animals, quickly increases to a maximal bright pink in 60–70 sec., when local swelling appears. During the next 4–7 min. the erythema fades and the swelling increases. Lesions 9–10 min. old have a brownish-red hue that persists for up to 2 hr. but disappears when the animals are exsanguinated.

The response almost certainly is mediated by liberated histamine. It is highly susceptible to local or intravenous antihistamines; and small amounts of histamine, adequate to induce the response, are demonstrable in perfusates of skin samples bearing sites previously heated *in vivo*.

Susceptibility to local antihistamine.—In blued animals, at skin sites previously receiving an intracutaneous injection of 0·1 ml. 0·85 per cent saline, the immediate response induced by heating at 54° for 5 sec., is similar to that at untreated sites (Fig. 2), so that the effects of locally applied drugs can be tested.

The high susceptibility of the response to antihistamine is readily demonstrated at sites previously treated with the drug. In our hands, the preferred antihistamine in guinea-pigs was triprolidine (Mason and Wilhelm, unpublished; see Table I). Responses of colour intensity ++ to +++ at sites previously injected with saline are consistently decreased to intensity ± at sites injected with 2 μg. triprolidine in 0·1 ml. saline; and even 0·02 μg. triprolidine has a substantial effect (see Fig. 1, Wilhelm and Mason, 1958). The drug must be intracutaneous; the application to the skin immediately after heating of pieces of lint, 1 cm. square, soaked in 1 per cent triprolidine solution does not affect the response.

Susceptibility to intravenous antihistamine.—The interpretation of the local effects of antihistamines, including triprolidine (Green, 1953) is complicated by their non-antihistaminic side-actions *e.g.* anticholinergic and anaesthetic effects. To estimate this side-activity, we tested the effects of small doses (0·1–1·0 mg./kg.) of various antihistamines given intravenously. Of nine antihistamines tested (Table I), each suppressed the immediate response in proportion to its relative potency in decreasing the permeability response to injected histamine. Since these relative potencies ranged from 0·7 to 1750, this is good evidence that suppression in all cases is a specific antihistamine effect.

Demonstration of liberated histamine at the heated site.—Three immediately adjacent skin sites on one side of the back of the trunk were heated at 54° for 5 sec., the animal killed and the skin, 3 × 1 cm., excised. Perfused by the technique of Miles and Wilhelm (1958), the skin samples yielded 0·015–0·03 μg. histamine; whereas unheated samples from corresponding sites on the other side yielded no detectable histamine in 2/4 experiments, trace amounts that were not estimable in a third, and 0·007 μg. in a fourth. The amounts liberated by heating are small, and we have no information about the residual histamine after perfusion; but taking the results at face value, the yield from 3 lesions of 0·015–0·03 μg. free

TABLE I.—*Inhibition of the Permeability Responses to Histamine and to Heating at 54° for 5 sec. by Various Antihistamines Given Intravenously (0·1 mg./kg.) in Guinea-pigs*

Antihistamine	Factor of inhibition* of response to: Histamine	Factor of inhibition* of response to: 54° for 5 sec.
Triprolidine[1]	1750	120
Chlorprophenpyridamine[2]	530	21
Mepyramine[3]	50	16
Promethazine[3]	12	7
Tripelennamine[4]	6	15
Chlorcyclizine[1]	1·6	3
Diphenhydramine[5]	1·4	4
Antazoline[4]	1·1	1·3
Thenalidine[6]	0·7	1·6

* Estimated for histamine from dosage-response lines; for heating from colour-intensity expressed in terms of local concentration of dye, from the data in Fig. 1.

[1] Burroughs Wellcome; [2] Allen & Hanburys; [3] May and Baker; [4] Ciba; [5] Parke Davis; [6] Sandoz.

histamine represents an order of dosage adequate to induce the superficial blueing of the lesions, 9 mm. diam., elicited in thermal injury (see Sparrow and Wilhelm, 1957). Furthermore, the results are consistent with those obtained by testing the liberation of histamine by *in vitro* heating (see below).

In summary, the immediate permeability response in guinea-pigs is induced by liberated histamine. However, this response to thermal injury, like that to histamine injected intracutaneously, lasts only about 10 min.

The delayed response

More prolonged responses to thermal injury have been reported. Thus Sevitt (1958) observed pronounced local oedema, maximal 8 hr. after heating skin sites at 60° for 10 sec. in anaesthetized guinea-pigs. Accordingly, we investigated the effects of heating at 54° for periods longer than 5 sec. in our unanaesthetized animals, and elicited a characteristic biphasic permeability effect.

Heating at 54° for 20 sec. in previously blued guinea-pigs induces an immediate response less pronounced than that induced by heating for 5 sec., and which is represented by a ring of colour 1–2 mm. wide, ++ to +++ intensity, at the periphery of the site. In duration of effect and susceptibility to triprolidine, the ring response is similar to that induced by 54° for 5 sec. After ½–1 hr., blueing appears within the peripheral ring, and the entire lesion becomes evenly coloured (++ to +++ intensity) during the next 2–3 hr.

The maturation of this delayed response is summarized in the left-hand figure of Fig. 3. In this experiment, intravenous dye was given to guinea-pigs bearing skin sites heated at 54° for 20 sec. 1 min. to 30 hr. previously. The immediate response (A in Fig. 3) subsides in 10 min.; the delayed response (B) appears in ½–1 hr., is maximal in 3–4 hr., and declines in 6–8 hr.

The curious absence at the centre of the lesion of the immediate blueing effect when heating is increased from 5 to 20 sec. requires an explanation. The absence of blueing cannot be due to diminished supply of circulating dye by reason of any

long-lasting obstruction to the blood supply, *e.g.* by intravascular coagulation, because the delayed response later appears in the same areas; nor is there any temporary obstruction, because the blood vessels in freshly heated skin sites readily fill with carbon suspensions injected, at pressures not exceeding 80 mm. Hg, into the artery (axillary) supplying the area of skin. Sites heated at 54° for 20 sec. fill as readily in rate and intensity, both at their centre and periphery, as the surrounding skin; and the filling is uniform in sites 5 sec., 1½, 2, 5, 12, 25, 30 and 120 min. old at injection.

These results do not entirely exclude an early, transient and partial vascular spasm of a magnitude sufficient to lower the intra-capillary pressure and so decrease the exudation of dye. There is some support for this view in that early

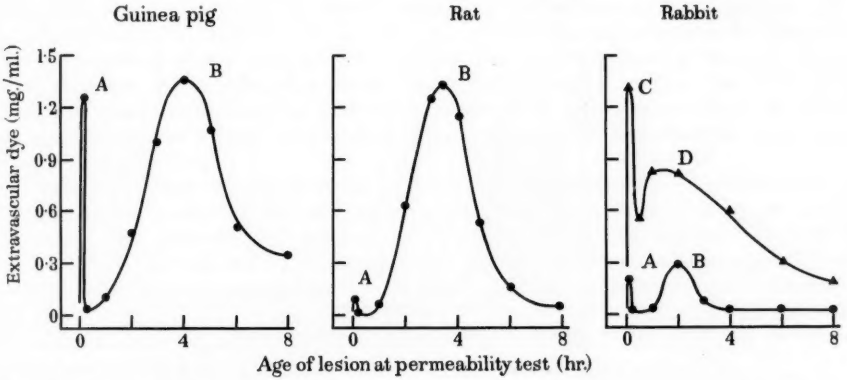

FIG. 3.—The biphasic permeability response to thermal injury in the unanaesthetized guinea-pig, rat and rabbit. The immediate response (A) is optimally induced by heating at 54° for 5 sec., the delayed response (B) by heating at 54° for 20 sec.

The response illustrated by curves C and D in the rabbit is that to heating at 56° for 20 sec.

blanching of the heated sites is observed; but it is unlikely that decreased blood flow is responsible for absence of central blueing since the blanching lasts only 30 sec. It is replaced by a reddish-brown colour that quickly reaches maximal intensity in a further 15–30 sec., persists for 5–10 min. and then gradually fades The macroscopic appearance of the lesion suggests venular dilatation and stasis. Accordingly, vascular spasm, if it in fact occurs, is very short-lived.

The absence of the central histamine blueing does not seem to be related to the prolonged refractory period that various permeability factors induce at the sites of their intracutaneous injection in guinea-pigs (Miles and Miles, 1952). Intracutaneous histamine and globulin PF, in doses that induce lesions 6–7 mm. diam. in untreated skin, each induce no blueing in heated sites 5 min. after heating, but are fully effective in sites 12 and 25 min. after heating. The absence of response in the lesion-centre therefore appears to be due to a temporarily induced insusceptibility of the vessels to various permeability factors, including histamine; but its nature is not clear.

There is a noteworthy distinction between the immediate and delayed responses

which may have a bearing on their pathogenesis. It is of great importance in optimally eliciting the two responses to test the appropriate area of skin of the dorsal trunk. The immediate response is reasonably consistent in intensity over the area of skin routinely used for testing permeability factors by intracutaneous injection (see Fig. 2). This area is bounded in the moderately extended animal of 700 g. by a transverse line immediately behind the posterior margins of the scapulae in front, and a parallel line through the lumbo-sacral junction behind ; and laterally by vertical lines 4 cm. on either side of the spine. In this area, the intensity of the immediate response increases progressively, although only moderately, from the lumbo-sacral junction towards the upper thoracic level.

Although this area of skin is reasonably homogeneous with respect to the response to 5 sec. heating, it is so heterogeneous with respect to 20 sec. heating that testing must be confined to a different and more restricted area of skin. The

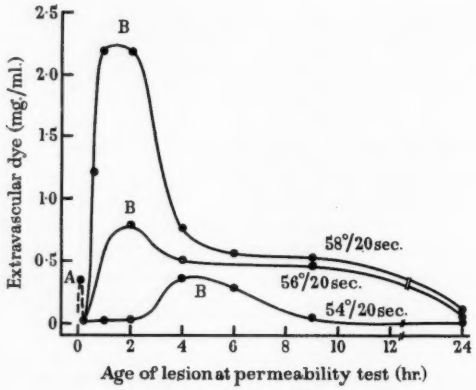

FIG. 4.—The accelerated appearance of the delayed response in the guinea-pig when the degree of heating is increased from 54° to 58° for 20 sec. (anaesthetized animal). In each case the immediate response (A) is confined to the periphery of the heated site.

delayed response to 20 sec. heating is strong in the cervical and upper thoracic area and declines caudally to become weak in the lumbar skin. The response is reasonably consistent in the area, on the moderately extended animal, bounded in front by a transverse line through the anterior margins of the scapulae ; and behind by a second line 7·5 cm. caudal and parallel to the first ; avoiding the skin 1 cm. on each side of the spine, the area extends laterally for 3·5 cm.

This area was used for all further investigations of the delayed response. The intensity of response is consistent even within this area, but variable results are obtained provided that the test sites in each experiment are randomized on the skin of 3–4 animals.

With temperatures higher than 54°, however, the response to heating in anaesthetized guinea-pigs again becomes consistent. Fig. 4 illustrates an experiment in which animals were blued at appropriate intervals after injury under anaesthesia. Each order of heating induces at the periphery of the test site an immediate response (shown by the broken line) susceptible to antihistamine. Heating

at 54° induces a delayed response maximal after about 4 hr. (cf. unanaesthetized guinea-pigs); but, at sites heated at 58° for 20 sec. in antihistamine-treated animals, the delayed response begins to appear in 5 min. and rapidly matures after 15 min. to become maximal in 1 hr. (cf. Sevitt, 1958).

At this stage we have concentrated on investigating the effects of milder heating and, unless stated otherwise our further tests of the delayed response were made in skin heated at 54° for 20 sec.

We have not as yet identified the mediator of the delayed response; our investigations were confined almost entirely to a study of the effects of antagonists of histamine, 5-hydroxytryptamine and proteases.

Insusceptibility to antihistamines.—The delayed response is unaffected by intravenous or local triprolidine, given before or during the maturation of the response.

Intravenous triprolidine, 0·1–1·0 mg./kg. inhibits the skin response to histamine 1500- to 2000-fold for at least 2 hr. Given just before inducing thermal injury, and repeated 2 hr. later, the same doses of triprolidine abolish the immediate response without affecting the 4 hr. delayed response. Triprolidine, 10 μg. in 0·1 ml. saline, injected in heated sites during the development of the delayed response is similarly ineffective.

Although these results strongly suggest that histamine has no preliminary or direct role in mediating the delayed response, this interpretation is by no means certain. The permeability response induced by histamine-liberators like 48/80 (Paton, 1951) is well known to be relatively insusceptible to antihistamine (Paton, 1957); and if a substantial portion of the histamine content of skin is liberated by heating at 54° for 20 sec., and subsequently is pharmacologically insusceptible to antihistamine, it might mediate the delayed response. Accordingly, we determined the liberation of skin histamine by *in vitro* heating.

Liberation of skin histamine by heat in vitro.—Discs of closely clipped skin 11 mm. diam. were excised from the usual test area in guinea-pigs, rats and rabbits. Batches of discs from partly randomized sites were weighed and incubated for 5 min. in Locke's solution (preparation A), maintained at 54°, 65°, 75°, 85° and 95° respectively. The heated discs then were minced as finely as possible with sharp scissors and leached in Locke's solution (preparation B) at 37° for 5 min. to recover liberated histamine not diffusing from the intact discs. The recorded results for B in Fig. 5 are corrected for histamine released in mincing the skin by subtracting that leached from minced discs after heating at 37°. Finally, the minced discs were boiled in Locke's solution (preparation C) for 5 min. to obtain any residual histamine.

The sum of A, B and C is designated total histamine content; in guinea-pigs, it amounted to 3·6–4·2 μg./g. skin, was similar to that of control discs minced and boiled (4·0 μg./g. skin), and to that of minced skin extracted with acid for histamine by the technique of Feldberg and Talesnik (1953). In rats, the sum of histamine from A, B and C was 9·1–16·2 μg./g. skin, which equalled the histamine extracted by boiling unheated skin and was 95 per cent that of acid extracted skin. The sum of histamine from A, B and C in albino rabbits (4·6–9·1 μg./g. skin) and in New Zealand red rabbits (2·5–2·8 μg./g. skin) equalled that from control preparations and was about 90 per cent that of acid extracted skin.

The histamine released in guinea-pig skin by *in vitro* heating at 54° (A + B in Fig. 5) is only about 10 per cent of the total. At higher temperatures diffusion of liberated histamine from skin discs increases with the temp. of heating (Rosenthal, Samet, Winzler and Shkolnik, 1957); but as much histamine is liberated at 65° as at 95° (A + B in Fig. 5). The percentage of histamine liberated by heating at 54°–65° is directly proportional to the temperature of heating.

Similar results were obtained with rat and rabbit skin (Fig. 5). In each, a higher percentage of histamine escapes from the intact heated discs (curves A) than from heated guinea-pig skin, leaving a correspondingly smaller amount recoverable by leaching the minced discs

(curves B). The recorded amounts on curve B for rat skin are comparatively low because the minced controls yielded 33 per cent total histamine.

These *in vitro* results support those from perfused skin heated *in vivo* in that only small amounts of histamine are liberated by heating at 54°. The heated skin sites, 9 mm. diam., each weigh approx. 0·16 g. when excised. In our guinea-pigs, with 2–4 μg. histamine/g. skin, each site therefore contains 0·32–0·64 μg., which represents some 10–20 standard or effective blueing doses (EBD ; see Miles and Wilhelm, 1955 ; Sparrow and Wilhelm, 1957). Even if all the skin histamine were liberated by heating at 54°, it would on this evidence be inadequate to induce and maintain increased permeability for some hours at the heated site, unless the local skin content of histamine is in some way increased ; such as by the accumulation at the injured site of histamine from elswhere (Geiringer and Hardwick, 1953), or augmentation by increased histidine decarboxylase activity, as Schayer, Rothschild and Bizony (1959) observed in rats treated systemically with 48/80.

Table II records the histamine content of skin sites excised 2 min.–5 hr. after heating at 54° for 20 sec. The amounts of liberated histamine, *i.e.* leached from minced skin, and the total histamine content of the skin are remarkably constant in sites 2 min. to 3½ hr. old ; and even the slight fall in total content in 5 hr. sites is similar to the lower limit of that in unheated sites.

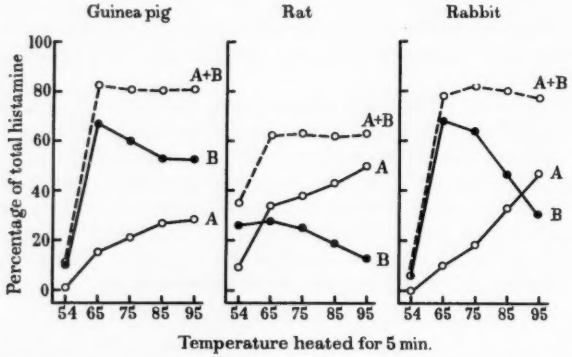

FIG. 5.—The liberation of diffusible histamine from the skin of the guinea-pig, rat and rabbit by *in vitro* heating.

A = histamine diffusing from intact discs of skin during 5 min. at 37°.

B = histamine diffusing under similar conditions from the same discs after mincing (less diffusible histamine released when unheated discs are minced).

Total histamine on the ordinate is that released from minced discs boiled for 5 min. (see text).

This aspect of the work clearly needs expanding, but to date we have no evidence that heating at 54° releases histamine sufficient to mediate the delayed response, or that thermal injury induces a local increase of available histamine at the injured site.

Insusceptibility to protease inhibitors.—The permeability effect of locally injected guinea-pig globulin PF, like that of the delayed response, is insusceptible to antihistamine. The globulin PF, however, is strongly suppressed by prior *in vitro* treatment with the trypsin inhibitors from soya bean (SBTI ; Worthington Biochemical Sales Co.) lima bean (LBTI ; Nutritional Biochemicals Corp.) and potato (PoTI) ; and with the esterase inhibitor, di-isopropyl phosphofluoridate (DFP ; see Miles and Wilhelm 1955, 1960*a* and *b* ; Wilhelm *et al.*, 1955, 1957 ; Becker, Wilhelm and Miles, 1959) ; but none of these preparations antagonized the delayed response.

TABLE II.—*Histamine Diffusing or Extractable from Guinea-Pig Skin Excised at Various Intervals after Local Heating* in vivo *at 54° for 20 sec.*

Expt.	Successive treatment of excised discs of skin in Locke's solution	Histamine (μg./g. skin) released from skin: Excised (min.) after thermal injury 2	30	90	210	300	Unheated control
1	Intact disc, 5 min. at 37°	0·02	0·01	0·02	0·01	0·02	0·02
	Minced disc, 5 min. at 37°	0·50	0·64	0·44	0·39	0·23	0·22
	Minced disc, boiled, 5 min.	4·77	4·46	5·09	4·51	3·73	4·91
	Total	5·29	5·11	5·55	4·91	3·98	5·15
2	Intact disc, 5 min. at 37° / Minced disc, 5 min. at 37°	0·21	0·31	0·34	0·27	0·28	0·22
	Minced disc, boiled, 5 min.	3·97	4·64	3·86	4·21	3·39	4·06
	Total	4·18	4·95	4·20	4·48	3·67	4·28

In groups of 3 guinea-pigs, 8 sites were heated on either side of the trunk of each animal ; 0·1 ml. volumes of a test preparation in saline were injected in sites on one side 0, 1, 2, and 3 hr. after heating ; and the sites on the other side, injected with 0·1 ml. saline, served as controls. The animals were blued 3½ hr. after heating. SBTI, LBTI, PoTI and the trypsin inhibitor in ovomucoid, each in concentrations of 100 μg./ml., and DFP, 10^{-3}–10^{-4} M, had no effect on the delayed response. It should be noted also that, injected before heating at 54° for 5 sec., none of these preparations affected the immediate response.

The permeability response to globulin PF is strongly suppressed by protease inhibitors injected locally with the PF, but little affected in animals previously given the inhibitors systemically. The maximum intravenous dose of SBTI, LBTI, or PoTI without non-specific effects on the permeability responses to histamine or other non-protease factors is 3·3 mg./kg. In this dose, SBTI suppresses the response to globulin PF only 4-fold, and has no effect on the delayed thermal response. Intravenous LBTI or PoTI are even less effective on globulin PF, and were not tested in thermal injury.

The effects of systemic DFP cannot be tested satisfactorily because intraperitoneal doses of 1·5 mg./kg. induce depression of the rectal temperature and considerable collapse in the guinea-pig.

The negative results obtained with protease inhibitors do not satisfactorily eliminate mediation by the globulin PF, because even the local effects of these inhibitor preparations *in vivo* are restricted in potency and duration. At sites injected with SBTI or PoTI, 10 μg. in 0·1 ml. saline, the suppression of immediately injected globulin PF is considerably less than when the inhibitor and PF preparations are mixed before injection ; and in 20 min. their suppressive effects further decrease considerably.

Demonstration of globulin PF at heated sites.—We have referred above to the demonstration by Miles and Wilhelm (1958) that the globulin PF and its native inhibitor in plasma are present in the tissue fluid of skin. Using their intercellular skin-perfusion technique, we found that perfusates of skin sites previously heated at 58° for 20 sec. contained more globulin PF than unheated sites. However, an increased concentration of plasma globulins in the tissues is an inevitable consequence of increased vascular permeability, whatever the nature and source of the mediator (see also Miles and Wilhelm, 1960*a*) ; so that our demonstration of a

quantitative increase of PF does not resolve the problem of whether the extravascular PF causes or is a result of the increased permeability.

Insusceptibility to EDTA–Na_2.—As early as 1887, Arnold considered that a decreased concentration of calcium ions in or near capillary endothelial cells led to increased vascular permeability. The role of calcium in this respect subsequently has been raised by Chambers and Zweifach (1940, 1947) and Spector (1957).

We tested the effect of injecting the metal-chelating agent disodium ethylene diamine tetra-acetate (EDTA–Na_2; British Drug Houses), 10 μg. in 0·1 ml. saline, into heated sites during the subsequent 3 hr. It had no effect on the delayed response. This dose of EDTA–Na_2 should be sufficient to chelate local divalent calcium ions, because it is more than 20 times the dose inhibiting the *in vivo* action of *Clostridium welchii* toxin (see Moskowitz, Deverell and McKinney, 1956) as manifested by the delayed permeability effect (Blaker and Miles, unpublished).

Insusceptibility to sodium α-naphthyl acetate.—Höller, Lindner and Stoklaska (1958) reported that sodium α-naphthyl acetate, 100–200 mg./kg. body weight, given subcutaneously $\frac{1}{2}$ hr. before and after inducing formalin oedema in rats' paws, suppressed the oedema for 24 hr. and particularly for 2–8 hr. after injury. However, 1–10 mg./kg. intraperitoneally (the maximum dose without non-specific effects in our guinea-pigs) given 30 min. before heating and again $2\frac{1}{2}$ hr. later did not affect the delayed response in thermal injury. In this respect, it is noteworthy that we estimated changes in permeability in blued animals, whereas Höller *et al.* investigated the suppression of oedema.

Thermal Injury in Rats

The pattern of permeability response induced by heating skin sites at 54° for 5–20 sec. is similar in rats (Fig. 3) to that in guinea-pigs, except that the immediate response in rats is very small (Wilhelm, 1959).

The immediate response

Heating at 50–56° for 5–20 sec. only occasionally induces faint local blueing, but regularly elicits erythema and swelling. The faint blueing that sometimes appears in 2–5 min. occurs too erratically to test its susceptibility to inhibitor preparations. Erythema appears in 20–30 sec. and is unaffected by intravenous triprolidine, 0·5–2·0 mg./kg. Swelling begins 1–2 min. after heating and is moderately suppressed by triprolidine.

A very small response in the rat is to be expected because, although rat skin contains about 5 times more histamine liberable by *in vitro* heating than guinea-pig skin, histamine injected intracutaneously has about 25 times less PF potency in the rat than in the guinea-pig (Sparrow and Wilhelm, 1957).

The delayed response

Heating at 54° for 15–20 sec. regularly induces a delayed response similar to that in guinea-pigs. It has the same time-course (Fig. 3), the same variation in intensity according to the anatomical position of the test site, and the same insusceptibility to the PF antagonists that were tested in guinea-pigs.

Insusceptibility to antihistamine.—The 6 hr. time course of the delayed response is unaffected by intravenous triprolidine, 0·5–1·0 mg./kg., given just before in-

ducing the first lesion and again 3 hr. later (Spector and Willoughby, 1958). Nevertheless, this evidence against histamine as the mediator of the response is less convincing than in the guinea-pig because intravenous triprolidine, 0·5–1·0 mg./kg., in the rat decreases the permeability response to injected histamine only about 40-fold.

Insusceptibility to a 5-HT antagonist.—The permeability response in rat skin to injected 5-HT is decreased about 175-fold by the 5-HT antagonist, 2-brom-D lysergic acid diethylamide (BOL 148; Sandoz Products Ltd.), 1 mg./kg. intravenously. The same dose of BOL 148 is given before inducing thermal lesions and again 2 hr. later does not affect the 4 hr. time-course of the delayed response (cf. Spector and Willoughby, 1958).

Liberation of skin histamine and 5-HT by heat in vitro.—When the minced skin of our rats was boiled for 5 min., 9–16 μg. histamine/g. skin and 0·3–0·4 μg. 5-HT/g. skin were released. The heated sites, 9 mm. diam., each weighed approx. 0·09 g. when excised. About 35 per cent of the histamine content was released by *in vitro* heating at 54° for 5 min. (A + B in Fig. 5) and about 15 per cent of the 5-HT content. The amounts of histamine and 5-HT liberated are too small to mediate the delayed response; and, judged by the PF potency of these preparations injected intracutaneously (Sparrow and Wilhelm, 1957), even the total content of histamine (1–2 EBD) or 5-HT (0·5 EBD) released by boiling in each site is insufficient to initiate and maintain the response.

Insusceptibility to protease antagonists.—Injected at hourly intervals in heated sites during the maturation of the delayed response, 0·1 ml. volumes in Locke's solution of SBTI, LBTI, PoTI, 162 μg./ml.; of ovomucoid, 6 μg./ml.; or of DFP, 5×10^{-4} M, each did not suppress the response, compared with that at control sites injected with Locke's solution.

In rats, as in guinea-pigs, the dosage of inhibitors tested locally is limited to that which itself does not increase permeability. Hladovec, Mansfeld and Horakova (1958) observed that the oedema and exudation induced in the hind paw of the rat by injecting a 10 per cent kaolin suspension was suppressed by various trypsin inhibitors given intraperitoneally in doses of 50 mg./kg. 20 min. before injury, again 6 hr. after injury and then daily for 4 days. In their work, SBTI, PoTI and pancreatic trypsin inhibitor (PTI) suppressed oedema and exudation particularly in the acute phase (first 5 hr.); ovomucoid trypsin inhibitor was ineffective. Despite the large dosage employed, the suppression of oedema by SBTI, PoTI and PTI, and not by ovomucoid is noteworthy, because globulin permeability factor has the same susceptibility to these inhibitors. However, we have already noted the non-specific suppression of a PF-like histamine in the guinea-pig by intravenous doses of SBTI greater than 3·3 mg./kg., and the non-effect of this dose on the delayed response.

Spector and Willoughby (1958) tested the effects of systemic mepyramine, sodium salicylate and DFP on the permeability response ½ hr. and 3 hr. after heating skin sites in rats at 55° for 27 sec. Mepyramine suppressed the ½ hr. response but not the 3 hr.; DFP (3 mg./kg. intramuscularly 30 min. before heating) and salicylate each suppressed the response at both intervals.

Systemic DFP, in our hands, is toxic for rats as for guinea-pigs, and non-specifically suppresses the response to all substances tested (Becker *et al.*, 1959). Since Spector and Willoughby (1958, 1959*b*) considered that DFP may, at least in part, suppress the activation of globulin permeability factor or other susceptible

mediators in the later stages of the response to thermal injury, we extended our tests of the effects of systemic DFP.

Rats given DFP, 1–3 mg./kg. intraperitoneally in arachis oil, become subdued in 10–20 min. In 15–30 min. some rats have collapsed. In 35–45 min. nearly all are passing soft offensive faeces and their rectal temp. has fallen 0–5° F. In guinea-pigs given DFP, 0·5 mg./kg., the rectal temp. falls 5–7° F. in 1 hr. compared with a fall of 0·8–1·2°F in controls receiving arachis oil; 0·1 mg./kg. has no effect.

Spector and Willoughby (personal communication) observed that in rats treated 3 days previously with DFP, the toxic effects of the drug had passed off, but that the delayed response to thermal injury still was suppressed. In our animals (Miles and Wilhelm, unpublished) given intraperitoneal DFP, 3 mg./kg., in arachis oil, many rats in 18 hr. have haemorrhagic crusts around the eyes and diarrhoea; a few die. In 24 hr. the survivors are considerably better and continue to improve for the next 2 days. By the third day, the rats are some 30 g. lighter in weight than before treatment; and their PF responses to rat globulin PF, histamine, 48/80 and 5-HT are in fact enhanced about 2-fold, compared with that in controls given arachis oil. In three such DFP-treated rats, the 6 hr. permeability effects of heating at 54° for 20 sec. were slightly greater than those in control animals.

In rats given DFP, 3 mg./kg. 30 min. before being anaesthetized for testing, the PF response to histamine is depressed 5-fold, that to globulin PF, 5-HT and polymyxin B (Burroughs Wellcome; see Bushby and Green, 1955) 2- to 3-fold. Spector and Willoughby (1959*b*) reported that DFP 2 mg./kg. given 30 min. before testing, suppressed the permeability effects in blued guinea-pigs, rats and rabbits of histamine, 48/80, 5-HT, substance P, and globulin factor. Accordingly, they concluded that "the effect of DFP on capillary permeability after burns might be due partly to a direct effect on capillaries leading to a general depression of permeability, and partly to inhibition of the activation of globulins or other permeability-increasing substances." Our own results do not permit any such firm conclusions; and we feel that until it is possible to make tests of this kind, in which the general toxic effects of DFP can be demonstrably excluded, any specific effect of the drug on permeability phenomena must be regarded as unproven.

At the suggestion of Dr. J. M. Barnes, we tested the effects of intravenous DFP, 4 mg./kg. given 15 min. after intraperitoneal eserine, 0·4 mg./kg., plus atropine, 2 mg./kg. Rats are killed in a few min. by this dose of intravenous DFP, but survive when protected by prior eserine-atropine treatment. In rats treated 4 hr. previously with DFP, the permeability response to globulin PF, histamine, 48/80 and 5-HT each are depressed about 2-fold, compared with those in eserine-atropine treated controls. In two further groups of rats similarly treated, the 4 hr. time-course of the delayed response to heating, begun 20 min. after giving DFP, was similar in each group, which in turn responded like untreated controls.

In summary, we have no evidence that DFP specifically decreases the delayed response; but the toxicity of local or systemic DFP in our hands prevented a satisfactory evaluation of its effect.

Insusceptibility to sodium α-naphthyl acetate.—As in guinea-pigs, sodium α-naphthyl acetate, 10 mg./kg. body weight, given intraperitoneally ½ hr. before heating and again 2 hr. later, did not affect the delayed response in rats.

Thermal Injury in Rabbits

Heating at 54° induces a biphasic permeability response in rabbits similar to that in guinea-pigs (Fig. 3).

The immediate response

In general, the immediate response is mild in intensity and irregular in appearance. It is elicited more consistently in albino than New Zealand red rabbits. In a few albino animals, 5 sec. heating provokes a response of intensity + to +±, that begins in 1 min., is maximum in 10 min., and almost completely suppressed by intravenous triprolidine, 0·1 mg./kg. In about half the animals, the colour-intensity of the response is ± to ± ; and in others, particularly red rabbits, the response is not elicited or has an intensity of only *trace* to ±.

Judged by the permeability response to histamine injected intracutaneously the drug has the same PF potency in both albino and red rabbits. The stronger immediate response to 5 sec. heating in albino than in red rabbits probably is associated with the higher histamine content of the skin of the albino breed (see above). However, the feeble response in most rabbits, compared with that in guinea-pigs, is surprising in view of the similarity of the skin histamine content in the two species, its ready release by *in vitro* heating (Fig. 5), and the high PF potency of injected histamine (Sparrow and Wilhelm, 1957) in the two species.

The delayed response

In both albino and red rabbits, 20 sec. heating induces a diphasic permeability response, similar in pattern to that in guinea-pigs, but feebler in intensity. The immediate response of about ± intensity is followed by a delayed response, ± to +± intensity, 2–4 hr. after injury (Fig. 3). The intensity of the delayed response varies at different sites even in the same animal ; but not as greatly as in the guinea-pig and rat. Nevertheless, the varying intensity, of what in any case is a feeble response, prevents satisfactory tests of its susceptibility to pharmacological antagonists.

A stronger and more reliable response is elicited by heating at 56° for 15–20 sec.—heating which in no way distresses unanaesthetized rabbits weighing at least 2·5 kg. Fig. 3 records the most frequent pattern of permeability change induced. A brisk response (C), that reaches a maximum colour intensity of ++± to +++ in 5 min., quickly declines to intensity +± and is succeeded by a second response (D) beginning after 30 min. and maximum in 1–2 hr. Peak C may be lower than D ; and the trough between C and D may be very shallow and occur 10 min. to 1 hr. after heating, or even be absent.

The standard pattern of response illustrated in Fig. 3 is not modified by intravenous triprolidine, 0·1–1·0 mg./kg. ; however, the response C is moderately suppressed by 1 μg. local triprolidine and strongly but not totally suppressed by 10 μg. These results suggest that the response D is not mediated by histamine, but is a delayed response appearing quickly after the higher degree of heating, as it does in the guinea-pig (Fig. 4).

Heating at 54°-56° for 5 min. *in vitro* liberates about 5 per cent of the 3–9 μg. histamine/g. rabbit skin (A + B, Fig. 5). Even the total content of 0·3–0·9 μg. in each site (approx. 0·11 g.) heated *in vivo* corresponds to only about 10–30 EBD (Sparrow and Wilhelm, 1957) ; and, as in the guinea-pig, seems insufficient to mediate the delayed response.

The response is not suppressed by locally injected SBTI, 10 μg. in 0·1 ml., or DFP, 0·1 ml. 10^{-4} M.

DISCUSSION

Our observation that thermal injury induces a biphasic permeability response —immediate and delayed—in the unanaesthetized guinea-pig, rat and rabbit confirms the reports of Sevitt (1958) in the guinea-pig, and of Spector and Willoughby (1958, 1959*b*) in the rat. Sevitt, like Spector and Willoughby, investigated the permeability changes induced in anaesthetized animals. Our study of the permeability responses to the minimal injury effective in unanaesthetized animals has established that the pattern and time-course of the diphasic response is similar in the guinea-pig and rat, as well as in the rabbit ; that, in terms of duration and intensity, the delayed response is the major one ; and it appears, in large part, to resolve the apparent discrepancies in the reported role of histamine as a principal permeability factor in thermal injury in man.

A feature of all known endogenous permeability factors is their wide variation in permeability-increasing potency according to the species of test animal (Sparrow and Wilhelm, 1957 ; Miles and Wilhelm, 1960*a*). This suggests that, if the mediators of natural inflammation are among these factors, they will not necessarily be the same in all species. On the other hand, the constancy of the second part of the biphasic permeability response in thermal, and in other types of injury (see below), suggests that a single factor is responsible ; though it does not at present appear to be one of the known factors. Apart from our inability to modify the delayed response with specified inhibitors of known factors, the prolonged time-course of the response is at variance with the 15–20 min. time-course of these factors determined experimentally. Miles and Miles (1952) have stressed this aspect of the problem ; and the shortcomings in this respect of the majority of known permeability factors are even more difficult to overlook because in at least one species, the rabbit, small amounts of its own endogenous factor—the globulin PF (Wilhelm *et al.*, 1958)—induce a prolonged permeability response.

The role of histamine in thermal injury

The clearly defined responses—immediate and delayed—elicited in guinea-pigs by heating at 54° tend to merge when skin sites are heated at 58°. The short-lived, but substantial, immediate histamine response elicited by heating at 54° is not only inhibited except at the periphery of sites heated at 58°, but also overshadowed by the strong delayed response appearing in 15 min. and insusceptible to antihistamine (Fig. 4).

Evidence of this kind from the guinea-pig seems relevant to experimental results in man and the rabbit, because in all three species histamine has high permeability-increasing potency (Miles and Wilhelm, 1960*a*). Accordingly, the results in guinea-pigs may adequately explain why mild heating at 43–52° for 5 sec. to 3 min. in man induces only a histamine-like response (Lewis and Love, 1926) ; whereas the permeability changes induced by heating at 60° for 15 sec. in man (Sevitt, Bull, Cruickshank, Jackson and Lowbury, 1952) or by 90° for 10 sec. in the rabbit (Weeks and Gunnar, 1949), are insusceptible to antihistamines. Nevertheless, our results in guinea-pigs differ from those of Sevitt (1958) in that he found no evidence that the immediate response in guinea-pigs was susceptible to antihistamine. From our own experience, the discrepancy probably is due to

different depilatory techniques. In our animals, the immediate response is maximal and consistent to heating at 54° for 5 sec., provided that no hair stubble is present at the test site. When depilation is incomplete, a higher temperature is required to elicit the response consistently, and hence is complicated by the early appearance of the delayed response.

The pattern of response to heating in our rats was similar to that described by Spector and Willoughby (1958, 1959*b*); but unlike us, they elicited what appears to be a response corresponding to our own immediate response in guinea-pigs and rabbits. They were able to suppress permeability changes in the first 30 min. by antihistamine, although as in our rats, not those of the maximal 1–2 hr. lesions.

In summary, we suggest that the immediate histamine response is minor, and is not a necessary precursor of the major delayed response; and that histamine itself does not elicit the delayed response. The weakness of our evidence is the absence of data concerning the effect of antihistamines on histamine liberated into a connective tissue matrix denatured by heating; or its effect on the additional histamine observed by Dekanski (1945) to accumulate rapidly in the skin of mice heated by immersion in water.

The role of proteases in thermal injury

The globulin PF does not owe its effects to the liberation of histamine. Accordingly, the globulin factor—which with its natural inhibitor is abundantly present in plasma, tissue fluid and lymph (Miles and Wilhelm, 1958), is readily activated (Mackay, Miles, Schachter and Wilhelm, 1953; Margolis, 1958) and has high permeability-increasing potency (Miles and Wilhelm, 1960*a*)—is a strong contender for the role of mediator of the delayed response.

The trypsin inhibitors from soya bean, lima bean and potato, as well as DFP, each strongly suppress the permeability effects of the globulin factor. These inhibitor preparations have considerable potency when mixed with the factor *in vitro* before testing; but given *in vivo*, their potency and duration of effect are restricted, and large systemic doses have non-specific depressive effects or are frankly toxic. Consequently the insusceptibility of the delayed response to these inhibitor preparations *in vivo* does not satisfactorily eliminate the globulin factor or other proteases, and hence polypeptides, as the mediators of the response. A satisfactory solution of the problem by the techniques we have used must await the discovery of low molecular, non-toxic protease inhibitors of high specificity.

Similarity of permeability changes in thermal injury to those in other varieties of injury

The pattern of biphasic permeability response in thermal injury is representative of that in various other types of injury. Experimental bacterial infection induces a biphasic response in the skin of guinea-pigs (Burke and Miles, 1958) and rats (Miles and Wilhelm, unpublished). The response in guinea-pigs has a similar time-course to that in thermal injury, but in rats the delayed response to *Staphylococcus aureus*, to *Streptococcus pyogenes* and to *Pseudomonas pyocyanea* takes 12 hr. to become maximal.

Petri, Csipak, Kovacs and Bentzik (1952) studied the effects of antihistamines on the vascular events in staphylococcal infection induced in rabbits' skin. They did not measure permeability changes in "blued" animals, but rather the accu-

mulation at the test sites of colloidal carbon given intravenously. Since the local accumulation of colloidal iron oxide at sites injected in guinea-pigs with histamine and with globulin factor has an onset and time-course similar to that of the permeability effects of these factors in " blued " animals (Wilhelm and Miles, unpublished), the results of Petri and his colleagues probably are valid for the associated permeability responses in infection. They observed a histamine response maximal in ½–1 hr. and absent in 6 hr., and a non-histamine response beginning in 1 hr. and maximal in 6 hr. Clearly the response seems similar in pattern to that in infection or thermal injury in guinea-pigs ; and similar in pharmacological mediation to that in thermal injury.

The α-toxin permeability factor in *Cl. welchii* type A toxin, injected intracutaneously in guinea-pigs induces a slight immediate response and a prolonged delayed response that begins in 1 hr., is maximal in 2–3 hr. and declining in 4 hr. (Elder and Miles, 1957). The delayed response to the toxins of *Cl. septicum* or *Cl. oedematiens* is similar in pattern but more prolonged.

Ultra-violet light also induces a diphasic response in guinea-pigs, rats and rabbits (Wilhelm, unpublished)—an immediate response that appears to be mediated mainly by histamine in guinea-pigs and rabbits, and by 5-hydroxytryptamine in rats ; and a delayed response, maximal in 20–30 hr., of which the mediator has not been identified.

On the other hand, the permeability changes in chemical injury induced by xylol, chloroform, turpentine and barium sulphide, like those to mild pinching, suggest that the response is monophasic and appears rapidly as in heating at 58° for 20 sec. (unpublished). Nevertheless the response appears to have an early and minor histamine element, suppression of which makes no substantial difference to the maximal response (cf. Spector and Willoughby, 1959*a*, on turpentine injury).

There is thus good evidence that the pattern of permeability response in thermal injury exemplifies that in mild injury in general ; so that inflammation induced by mild heating is a convenient and reliable working model for investigating the permeability changes in inflammation in general.

SUMMARY

The time-course and pharmacological mediation of increased capillary permeability induced by minimal thermal stimuli were investigated in the skin of the trunk of unanaesthetized guinea-pigs, rats and rabbits with pontamine blue circulating in their blood.

The general pattern of response is biphasic in all three species. In guinea-pigs, heating at 54° induces two distinct permeability responses—immediate and delayed. The immediate, best elicited by 5 sec. heating, appears in 1 min., and is maximal in 5 min. ; normal low permeability is restored in 10 min. The temporary immediate response is also elicited by heating at 54° for 20 sec., though much decreased in magnitude ; it is followed by a prolonged delayed response that appears in ½–1 hr., is maximal in 3–4 hr. and declining in 6–8 hr. The delayed response occurs progressively more quickly as the temperature of heating is increased to 58°.

In rats heating at 54° induced immediate and delayed responses like those in the guinea-pig. The immediate response, however, is very small or absent, although as in the guinea-pig, substantial local oedema appears.

In rabbits, an immediate response to heating at 54° appears inconsistently.

The delayed response is feeble, but has a time-course like that in guinea-pigs and rats. As in the guinea-pig, heating at a higher temperature (56° for 15–20 sec.) hastens the onset and increases the magnitude of the delayed response.

The immediate response in the guinea-pig and rabbit is mediated by histamine. Small amounts of free histamine sufficient to induce the observed response are recoverable from heated sites in guinea-pigs; and the response, both in terms of increased exudation of dye and observed oedema, in both species is abolished by low concentrations of local or systemic antihistamine. The local oedema that appears in the rat is only moderately suppressed by antihistamine.

The mediator of the delayed response was not identified. The response is not affected by antihistamine given during its maturation, or by antihistamine suppression of the preceding immediate response. Furthermore, the histamine content of heated sites in guinea-pigs does not increase during the course of the delayed response; and, in all three species, the amount of free histamine in the lesion induced by heating at 54° is insufficient to initiate and maintain the observed permeability effects during the response.

The delayed response is also unaffected by moderate local doses of the trypsin inhibitors in soya bean, lima bean and potato, and of the esterase inhibitor, di-isopropyl phosphofluoridate, all of which are strong antagonists of the globulin permeability factor *in vitro*. These negative results do not satisfactorily exclude the globulin factor as the natural mediator of the delayed response, because of the pharmacological limitations of the inhibitors *in vivo*.

In guinea-pigs and rats the response is insusceptible to local treatment with the trypsin inhibitor in ovomucoid, or with the metal-chelating agent, disodium ethylene diamine tetra-acetate; and to systemic sodium α-naphthyl acetate.

We are indebted to Philips Electrical Ltd., London for providing a Philishave Dry Shaver; to the Upjohn Company, Kalamazoo, U.S.A., for 5-hydroxytryptamine creatinine sulphate; to the Wellcome Research Laboratories, Tuckahoe, U.S.A., for compound 48/80; to Carnegies of Welwyn Ltd., Welwyn Garden City, for sodium α-naphthyl acetate; to the suppliers concerned for the antihistamines listed in Table I; to Dr. B. C. Saunders, University Chemical Laboratory, Cambridge, for di-isopropyl phosphofluoridate (DFP); and to Dr. J. Hladovec, Praha, Czechoslovakia, for potato trypsin inhibitor.

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STUDIES ON THE BIOLOGICAL PROPERTIES OF TWO PLAQUE VARIANTS ISOLATED FROM SE POLYOMA VIRUS

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RECENT communications report growth of SE polyoma virus in tissue cultures of various animal cells, its haemagglutinating property, and the induction of tumours in mice and hamsters (Stewart, Eddy, Gochenour, Borgese and Grubbs, 1957 ; Eddy, Rowe, Hartley, Stewart and Huebner, 1958*a* ; Eddy, Stewart and Berkeley, 1958*b*). The ability of SE polyoma virus to form plaques on mouse embryo cell cultures has also been recently reported (Eddy *et al.*, 1958*a* ; Dulbecco and Freeman, 1959 ; Winocour and Sachs, 1959). The present paper deals with the isolation of 2 kinds of plaques of SE polyoma virus characterized by differences in plaque size. In addition to this difference, the 2 strains derived from these 2 kinds of plaques have been found to differ widely in their haemagglutinating ability, cytopathogenic effect on mouse embryo cells and in their capacity to induce tumours in Swiss mice and hamsters.

MATERIALS AND METHODS

Virus.—SE polyoma virus sent by Drs. S. E. Stewart and B. E. Eddy to Dr. L. Sachs and given by him to us was employed throughout this study. Virus suspensions prepared from this material were kept at 4° while stock virus was kept frozen at −20° or in dried form in the refrigerator.

Cell cultures.—Primary cultures of mouse embryo cells were used for growing the virus. Decapitated Swiss mouse embryos from the 16–18th days of gestation were trypsinized in 0·12 per cent trypsin (Difco) solution in phosphate buffered saline (PBS). The trypsinization was carried out in flasks equipped with a magnetic stirrer. The trypsinized cells were collected and washed once and then diluted in lactalbumin hydrolysate medium containing 4 per cent calf serum at pH 7·4. A volume of 1 ml. containing 1×10^6 cells was used for tube cultures, 5 ml. for 5 cm. diameter plates and 12 ml. for flat bottles. The cultures were ready for use after incubation for 3–4 days, when a complete sheet of cells was formed. Before inoculation of the cultures with virus the medium was changed to Eagle's medium (Eagle, 1955) containing 5 per cent inactivated horse serum at pH 7·4–7·6. A 2 ml. volume of this medium was put in each tube and 10 ml. in each bottle ; no medium changes were made during a period of 2–3 weeks observation.

Animal tests.—One-day-old Swiss non-inbred mice and 3–6-day-old Syrian hamsters (*Cricetus auratus*) were used for virus inoculations, and the animals were inoculated subcutaneously with 0·2 ml. of virus suspension and kept up to 6 months under observation for development of tumours, deaths, and antibody production.

Antisera against the virus were obtained by bleeding the animals at various times following inoculation of live virus suspensions. Rabbit antisera were prepared in animals weighing 2 kg. by either 1st injection of 5 ml. virus suspension given intraperitoneally and 4 subsequent injections of 0·5 ml., 2 ml. and 2·5 ml. given intravenously with intervals of 10 days between each injection, and blood drawn one month later, or 20 ml. of virus suspension given intraperitoneally and blood drawn one month later.

It was found that sera received by using the second method contained live virus and therefore could not be used for neutralization tests.

Virus assay.—In order to determine virus titres, 3–4 tubes per log dilution were inoculated and used for end-point calculations according to the method of Reed and Muench (1938).

The tubes were observed for the cytopathogenic effect (CPE) of the virus on the cells on the 9th–10th day after inoculation and final readings were made on the 14th–16th day ; strong and slight CPE could be observed and were designated accordingly. These differences in the intensity of the CPE were found to be an important characteristic of the virus strains and will be dealt with later.

Infectivity titres were also determined by measuring the haemagglutinins (HA) in each inoculated tube on the 21st day after inoculation when the tests were completed. After preliminary studies (Gotlieb and Leventon, unpublished) the following method for detection of HA was used : 0·1 ml. of supernatant was removed and treated with 0·15 ml. of 0·01 N $NaIO_4$ at 37° or 44° for 30 min. and 0·15 ml. of 40 per cent glucose solution added to neutralize the periodate. Serial 2-fold dilutions were made in phosphate saline at pH 7·2–7·4 ; 0·5 ml. of 0·4 per cent guinea-pig red blood cells were added to 0·5 ml. of virus dilution and the test read by the pattern method after overnight settling of the cells at 4°.

For the haemagglutination-inhibition (HI) test, sera were inactivated at 56° for 30 min. and 2-fold serial dilutions were made in phosphate buffer saline. To 0·25 ml. of appropriate serum dilution, 0·25 ml. of 4–8 HA units of virus were added and incubated together for 1 hr. at 37°. To this 0·5 ml. of red blood cells were added and the mixture kept at 4° and read as indicated above. In order to remove non-specific HA and serum inhibitors, sera for the HI test were pre-treated with 1 : 10 RDE solution (N.V. Philips Roxane, " Duphar " Amsterdam, Holland) at a 1 in 10 serum dilution. Five parts of RDE were incubated with 1 part of serum overnight at 37° and then heated to 56° to inactivate the RDE.

Neutralization tests in tube cultures were carried out by inoculating 4 tubes with 0·2 ml. of a mixture of a standard amount of virus and appropriate serum dilution. Before inoculation, the mixtures were incubated for 1 hr. at 37°. Serum titres were based on 50 per cent tissue culture end-point calculations.

Virus assays on plates were carried out as follows : the tissue culture plates were washed once with Hanks' solution and inoculated with 0·3 ml. of virus suspension and incubated at 37° for 1 hr. for adsorption. Then the cultures were washed $\times 3$ to remove non-adsorbed virus and overlayed with a mixture composed of 4 ml. of 2 per cent agar and 4 ml. of Youngner's medium (1956) modified to contain 10 per cent horse serum and 0·05 per cent lactalbumin. On the 12–14th day of incubation 0·5 ml. of 0·05 per cent neutral red solution was added and the number of plaques read after 6 hr. at 37°. All plates were incubated in a 5 per cent CO_2 incubator.

Since pH, and the bicarbonate concentration of the medium had a marked effect on the CPE of the virus and on the production of HA, media with 2 different bicarbonate concentrations were used as well as with different pHs. The details will be given later.

RESULTS

Isolation and differentiation of the two strains

The SE polyoma virus was carried in mouse embryo cell culture tubes for 10 passages. Tests for HA, CPE and results of animal inoculations were found to be in accord with those described for SE polyoma virus (Stewart *et al.*, 1957 ; Eddy *et al.*, 1958*a* ; Eddy *et al.*, 1958*b* ; Eddy *et al.*, 1958*c* ; Stewart, *et al.*, 1958). At the 10th passage, plating of the material was carried out. Plaques 1–4 mm. diameter appeared on the 11th–14th day after inoculation. Two kinds of plaques differing in size were observed and isolated. On subsequent plating out, pure populations of large and small plaques could be obtained. The small plaques were of an average size of 1–1·5 mm. in diameter while the large plaques were 3–4 mm. in diameter. Fig. 6 shows the appearance of large and small plaques at various virus dilutions. In the small plaques a necrotic centre was more obvious when observed under the microscope while the large plaques were more diffuse and not all the cells at the site of the plaque were destroyed. From these 2 kinds of plaques, 2 lines of virus were carried and further studied for their various properties *in vitro* and *in vivo*. In Table I, the 2 strains are compared as to their plaque sizes, intensity of CPE, HA titres and their infectivity as measured

TABLE I.—*Characteristics of Two Strains of SE Polyoma Virus*

Strain No.	Characteristics of strains: Plaque size (mm.)	CPE*	HA titre/0·1 ml.†	Infectivity titre (ID_{50}/0·1 ml.) as measured by: CPE	HA	Strain designation
5552	1·0–1·5	+++	0	6·5	3·0	$C^+ H^-$
4671	1·0–1·5	+++	80	6·0	4·0	
7753	1·0–1·5	+++	240	6·0	4·5	
5551	5–4	+	960	3·5	5·7	$C^- H^+$
6781	5–4	+	3840	4·5	6·2	
8192	5–4	+	1920	5·5	6·0	

* + = slight.
+++ = strong.
† HA titres are expressed as the reciprocal of the highest dilution showing partial haemagglutination.

by CPE or HA. It will be seen from Table I that various fluids derived from the 2 kinds of plaques differed in their CPE in tubes and HA titres. The 3 fluids obtained from small plaques were highly cytopathogenic and had low HA titres, ranging from 0–240. On the other hand, the 3 fluids derived from large plaques were only slightly cytopathogenic and exhibited very high HA titres in the range of 960–3840. Fig. 7 shows the differences in the intensity of the CPE between the 2 strains. Accordingly the two strains were designated $C^+ H^-$ and $C^- H^+$. The infectivity titres of each of the 2 strains was measured according to its CPE and HA characteristics; the highest titre obtained, either by CPE or HA was subsequently used for the 50 per cent end point-calculations.

In order to determine whether the strains vary in their antigenicity, cross HI and neutralization tests were carried out using mouse and rabbit immune sera. The results of the cross HI tests are shown in Table II and those of the cross-neutralization in Table III. In comparison, results obtained with the parent SE polyoma strain are included. Only slight antigenic differences were found in the cross HI titres between the 2 virus strains as well as with the parent SE polyoma strain with mouse immune sera. This was also true for the cross neutralization tests. Essentially similar results were obtained in the cross HI tests using rabbit antisera; thus, it appears that the 2 strains derived from the parent SE polyoma virus did not change in their antigenic characters.

Growth curves

Growth curves of the 3 strains were carried out in bottles; a group of 14–16 bottles with mouse embryo cells were used after a complete sheet of cells was formed. Before seeding with virus, the sheet was washed once with Hanks'

TABLE II.—*Cross HI Titres of the Two Strains of Virus with Mouse Immune Sera*

Type of strain	HA units used	HI titre of pre-immunization serum	HI titre of antiserum against: $C^+ H^-$	$C^- H^+$
$C^+ H^-$	4	4,000	16,000	8,000
$C^- H^+$	4	4,000	24,000	32,000
Parent SE polyoma	4	2,000	16,000	16,000

TABLE III.—*Cross Neutralization of the Two Strains Against Mouse Antisera*

Type of strain	Number of ID_{50} used	Serum dilution end-point of: Pre-immunization serum	Antiserum against: $C^+ H^-$	Antiserum against: $C^- H^+$	Antiserum against: Parent SE polyoma
$C^+ H^-$	100	100	3,200	1,600	1,600
	10,000	100	400	200	100
$C^- H^+$	100	100	3,200	3,200	3,200
	10,000	100	200	200	300
Parent SE polyoma	100	100	3,200	1,600	1,600
	10,000	100	N.D.	100	100

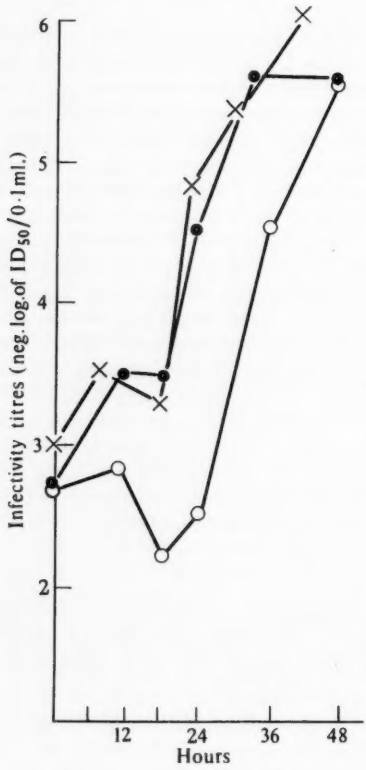


FIG. 1.—Growth curves of the 3 strains of SE polyoma during the first 48 hr. period following inoculation.

Parent strain ●——●
$C^+ H^-$ ×——×
$C^- H^+$ ○——○

solution and 0·5 ml. of virus inoculated. After 1 hr. of adsorption at 37°, the inoculum was drawn out and the tissue washed ×3 with prewarmed Hanks' solution and 10 ml. of Eagle's 5 per cent horse serum added. Infectivity titres of the seed virus and of virus left following adsorption were determined. Two bottles were trypsinized at the beginning of each experiment and the average cell count was used for calculating the multiplicity of infection. Most of the growth curves were carried out with multiples of about 1–2 TCD_{50} per one cell. One bottle each was withdrawn from the incubator at the desired time and the fluids

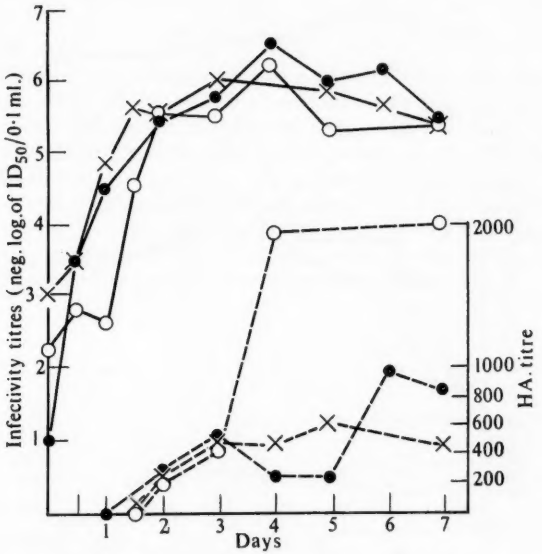


FIG. 2.—Infectivity and HA titres of the 3 strains of SE polyoma during a 7-day period of growth as measured by titration of the fluids.

titrated for HA and infectivity, and the cells counted. In some experiments, HA and infectivity titrations of the virus in the cells were also carried out. The sheet of cells was washed ×3 with Hanks' solution and then trypsinized and the live cells counted. The cells were disrupted by freezing (in a mixture of dry CO_2 and alcohol) and thawing 7 times; and the virus released from the cells was titrated.

Infectivity titres (ID_{50}) were calculated differently for each strain according to its CPE and HA characteristics. The CPE and HA end-points of the parent strain were approximately the same, while with the C^- H^+ and C^+ H^- strains, the highest titres either by HA or CPE of each strain respectively were used for the ID_{50} calculations. The growth rates of the 3 strains during the first 48 hr. are

shown in Fig. 1. It can be seen that with all 3 strains, virus starts to appear at 6–12 hr. after inoculation, and following a slight decline in titre, there is a further logarithmic rise up to 48 hr. Strain C^- H^+ seems to exhibit a more prolonged latent period than the other 2 strains. In Fig. 2, growth curves of the 3 strains during a prolonged period of time are presented. Here, the infectivity titres of the 3 strains are compared with their respective HA titres. It can be seen that maximum infectivity titres are reached on the 3rd day and afterwards level off

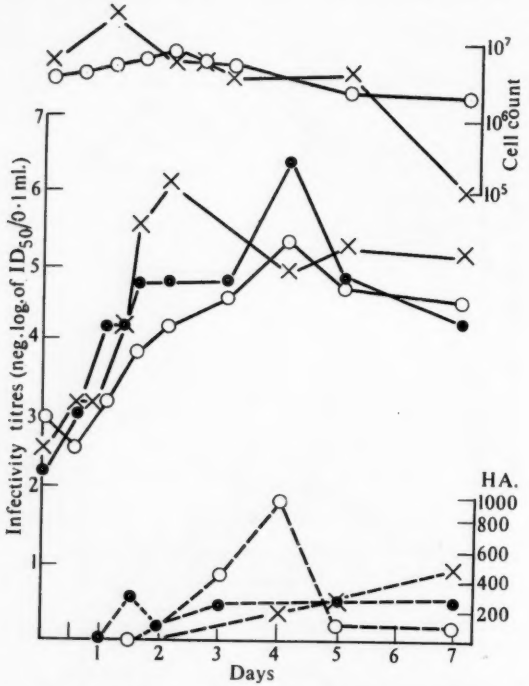
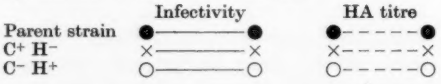


FIG. 3.—Infectivity and HA titres of the 3 strains of SE polyoma during a 7-day period of growth as measured by titration of virus inside the cells.



up to the 7th day. Essentially the infectivity growth curves run parallel in all 3 strains. The 3 strains differ, however, very markedly in their HA titres. Strain C^- H^+ stands out by its high HA titre reaching values of 1 in 2000 and higher from the 4th day onwards. The other 2 strains show markedly lower HA titres, especially for strain C^+ H^-.

Fig. 3 gives the infectivity and HA titres of the 3 strains in the cells, and the cell counts during a period of 7 days. It can be seen that with C^+ H^- strain, virus accumulates gradually in the cells and reaches its peak some time before it

TABLE IV.—*Effect of pH of Medium on HA Titres of the Three SE Polyoma Virus Strains*

Strain type	Strain No.	pH of medium	HA titre/0·1 ml.
$C^+ H^-$	7380	7·6	40
	7404	6·8	0
$C^- H^+$	7387	7·6	2560
	7409	6·8	40
Parent SE polyoma	7370	7·6	240
	7393	6·8	0

does in the other 2 strains. With all 3 strains, the HA titres in the cells are lower than in the fluids. The $C^- H^+$ strain shows an early rise and an abrupt fall in HA corresponding to the rise in HA in the fluid. In the upper part of Fig. 3, the cell counts are shown, and it may be seen that on the 7th day the cells, in which the $C^+ H^-$ strain was growing, were mostly degenerated, while with the $C^- H^+$ strain the cell count declines more slowly. With the latter strain, we were able, by changing the medium every 2–3 days, to keep the cell culture almost intact for 50 days and the cells continued to release virus with a titre of $10^{-5.5}$–$10^{-6.5}$ per 0·1 ml. when measured at every medium change.

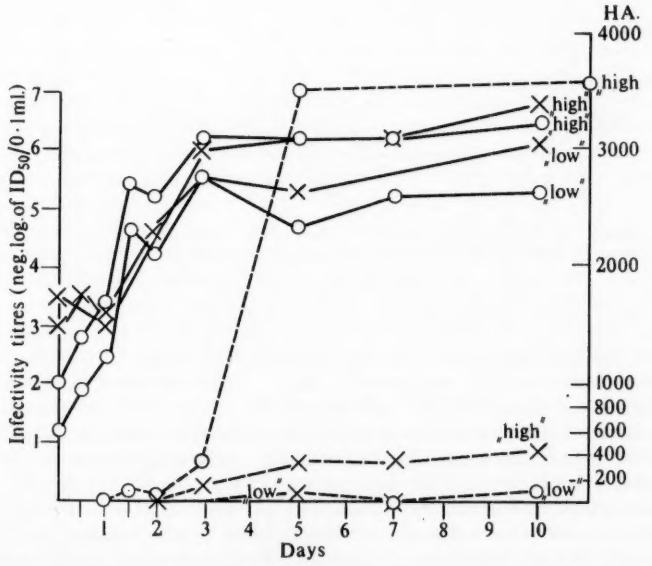


FIG. 4.—Influence of "low" and "high" bicarbonate concentrations on the infectivity and HA titres of the 2 plaque variants, as measured by titration of the fluids.

	Infectivity	HA titre
$C^+ H^-$	×———×	×– – – –×
$C^- H^+$	○———○	○– – – –○

Effect of pH and $NaHCO_3$ on the CPE and HA titres of the C^- H^+ strains

It was observed early in our work that the CPE and HA titres of the 2 strains were influenced by pH and bicarbonate concentration of the medium as was also reported recently by Eddy and Stewart (1959). In order to test the effects of changes in pH and bicarbonate concentrations, modifications were introduced in Eagle's medium so as to obtain media with differing pH and bicarbonate concentrations. The pH ranged from 6·8 to 7·6. Differences in bicarbonate concentrations were obtained by varying the amounts of bicarbonate added. To

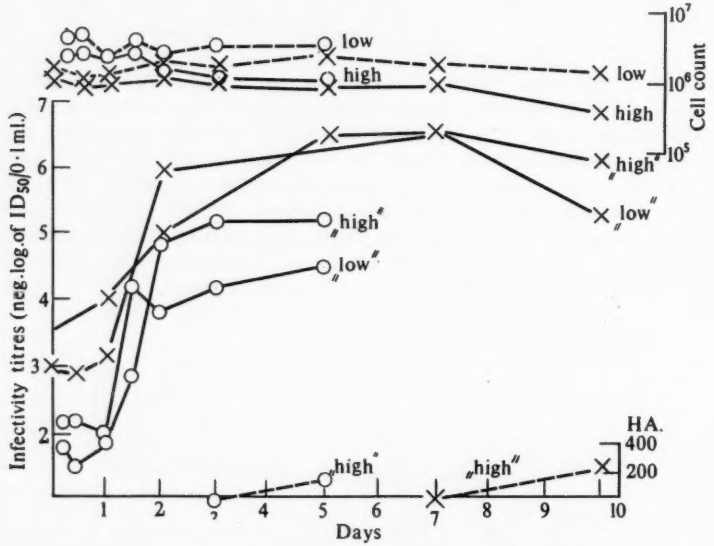


FIG. 5.—Influence of "low" and "high" bicarbonate concentrations on the infectivity and HA titres of the 2 plaque variants as measured by titration of virus inside the cells.

	Infectivity	HA titre
C^+ H^-	×——×	×- - - - - ×
C^- H^+	○——○	○- - - - - ○

1 l. Eagle's 0·55 g. $NaHCO_3$ was added and called "low"—and to 1 l. Eagle's 2·75 g. $NaCHO_3$ was added and called "high" bicarbonate concentrations.

Table IV shows the effects of 2 pH concentrations on HA titres of the 2 strains. It will be observed that very low or no haemagglutinins were formed in acid pH, while various HA titres were obtained for the different strains in alkaline pH. The influence of pH was most conspicuous with the C^- H^+ strain, in which maximal HA titres were obtained at alkaline pH and very low at acid pH.

In order to test the influence of the 2 bicarbonate concentrations on the infectivity and HA titres of the C^- H^+ and C^+ H^- strains, growth curves were made in the usual manner employing "low" and "high" bicarbonate concentrations. ID_{50} and HA titres were determined for both the supernatant and cells.

The results of these experiments are shown in Fig. 4 and 5. It will be observed

that there is a difference of about one log in the infectivity titres of each of the 2 strains when grown in media of " high " and " low " bicarbonate concentration. The most striking effect of bicarbonate concentration was in the HA titres. This is especially evident for the $C^- H^+$ strain in which " high " bicarbonate concentrations resulted in a great rise in HA titres whereas " low " bicarbonate suppressed almost completely production of haemagglutinins. The HA titres of the $C^+ H^-$ strain were low at " high " bicarbonate concentration and nearly none were formed in medium containing " low " bicarbonate. Although it is realized that this may have been affected by the differences in infectivity titres at " low " and " high " bicarbonate concentrations, it cannot wholly account for such a wide divergence in the HA titres. Fig. 5 shows the influence of " low " and " high " bicarbonate concentrations on the infectivity and haemagglutinin contents of the cells. These results are correlated with the respective cell counts. No or very low HA titres can be detected inside the cells, at both bicarbonate concentrations of the 2 strains, in contrast to the large amounts of haemagglutinins released into the fluids when the " high " bicarbonate concentration was used (Fig. 4). The bicarbonate concentration seemed to have a great influence on the cell counts ; with both strains, low bicarbonate concentrations resulted in better maintenance and longer survival of the cells.

Animal tests

When the tissue culture fluids of the 2 strains were inoculated in one-day-old mice or in infant hamsters, a difference in the tumour-inducing capacity of the 2 strains was observed. This was especially evident in regard to the percentage of animals developing tumours or dying as well as in the time of appearance of the tumours after inoculation and their site. The difference in response to inoculation with the 2 strains was more conspicuous in mice than in hamsters. Table V demonstrates condensed data of our experiments. For comparison, results of animal inoculations of the parent SE polyoma strain are included.

It can be seen from Table V that strain $C^- H^+$ induced a significantly higher percentage of tumours than the $C^+ H^-$ strain. This was evident in many experiments carried out with the 2 strains. Following inoculation of the $C^+ H^-$ strain, tumour development was retarded up to 5–6 months after inoculation. The $C^- H^+$ strains induced tumour formation regularly in up to 100 per cent of the animals. Most of these tumours appeared up to 4 months following inoculation as indicated in Table V. The behaviour of the parent SE polyoma virus seemed to be closer to the $C^- H^+$ strain in this respect. Mice dying of unknown reason were not included in the calculations since it was not always possible to determine the exact cause of death. In addition to the difference in the tumour inducing capacity, the $C^- H^+$ strain showed a high percentage of animals with haemorrhages in the lungs and with paralysis of the hind legs due sometimes to visible tumours in the spinal cord. On the other hand, the $C^+ H^-$ strain was characterized by limited tumour formations mostly of the mammary and lymph glands.

The second part of Table V contains the comparative results of inoculations of these strains in infant hamsters. Owing to the high susceptibility of infant hamsters to polyoma virus (Eddy *et al.*, 1958*c* ; Rowe *et. al.*, 1959) titrations of the various strains in hamsters were carried out in order to enable better differentiation. Strain $C^- H^+$ could be readily titrated in hamsters showing a diminishing effect in the animals inoculated with the various dilutions of virus. The per-

TABLE V.—*Tumour Development in New-born Mice and Hamsters Following Inoculation with Three Strains of Polyoma Virus*

Strain type	Inoculation dilution	Number of animals inoculated	Number of animals developing tumours during (months) 1–4	5–6	Death reason unknown	Percent of tumour development
		Mice				
$C^+ H^-$	Undiluted	16	—	—	—	0*
	10^{-1}	30	—	5	4	19
$C^- H^+$	Undiluted	19	10	1	2	65
	10^{-1}	21	14	—	4	82
Parent SE polyoma	Undiluted	25	12	6	—	72
	10^{-1}	5	5	—	—	100
	10^{-2}	6	1	—	—	16
	10^{-3}	6	1	1	—	32
	10^{-4}	5	2	1	—	60
	10^{-5}	4	1	1	—	50
	10^{-6}	7	—	—	—	0
		Hamsters				
$C^+ H^-$	Undiluted	7	4	2	—	85
	10^{-1}	2	1	—	—	50
	10^{-2}	4	—	2	—	50
	10^{-3}	3	—	1	—	33
	10^{-4}	4	1	—	—	25
	10^{-5}	5	—	—	—	0
$C^- H^+$	Undiluted	9	9	—	—	100
	10^{-1}	4	4	—	—	100
	10^{-2}	5	—	4	—	85
	10^{-3}	4	1	1	—	50
	10^{-4}	7	—	3	—	43
	10^{-5}	6	—	1	—	18
	10^{-6}	4	—	—	—	0
Parent SE polyoma	Undiluted	11	11	—	—	100
	10^{-1}	5	2	3	—	100

* Observation discontinued after 4 months.

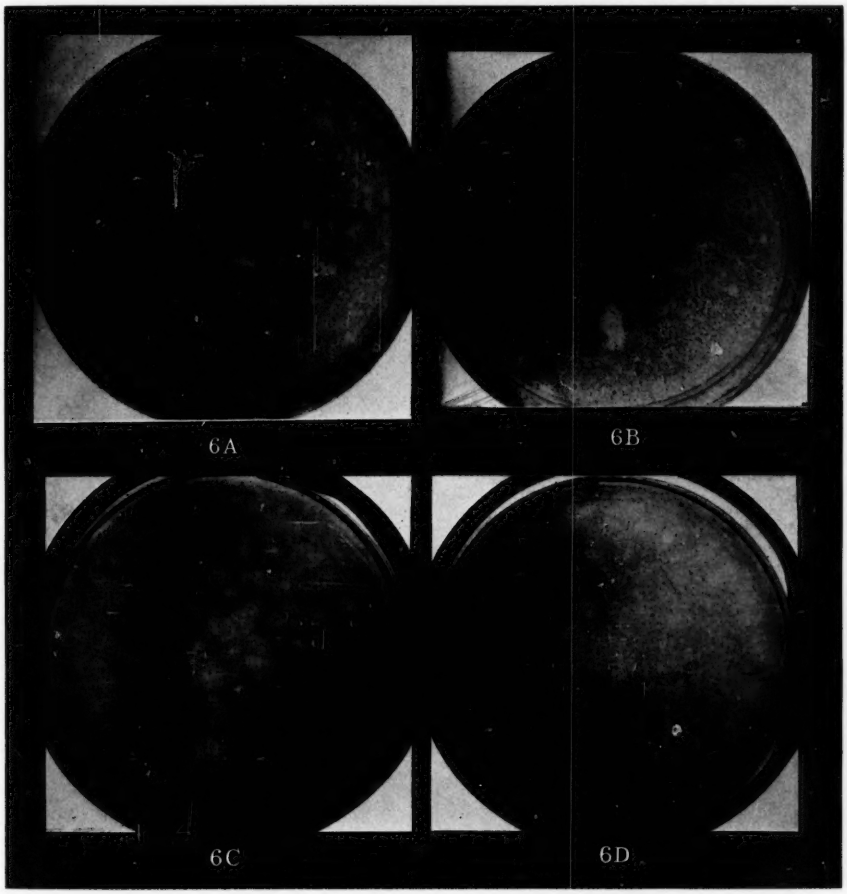
EXPLANATION OF PLATES

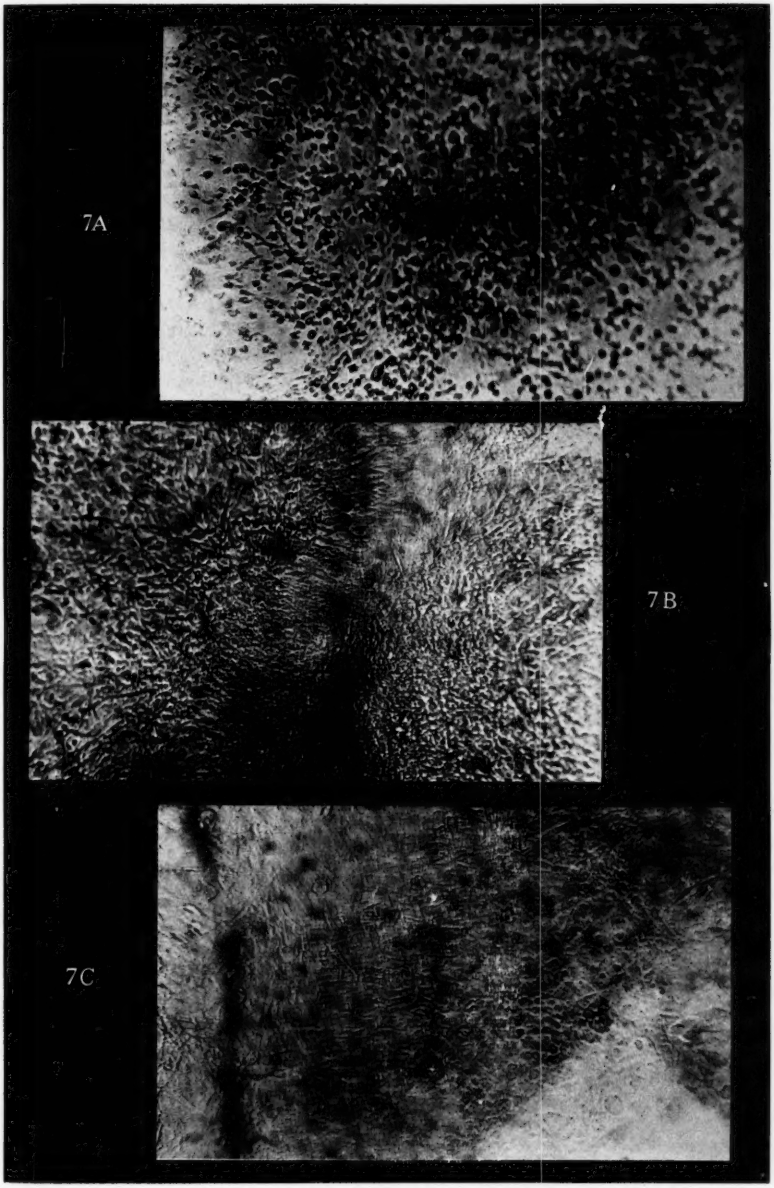
FIG. 6.—Plaques of $C^+ H^-$ and $C^- H^+$ strains in mouse embryo cell culture plates.

A. Strain $C^+ H^-$, dilution 10^{-3}.
B. „ $C^+ H^-$, „ $10^{-3.5}$
C. „ $C^- H^+$, „ $10^{-4.5}$
D. „ $C^- H^+$, „ $10^{-5.5}$

FIG. 7.—Differences in cytopathogenic effect between $C^+ H^-$ and $C^- H^+$ strains in mouse embryo cell culture tubes; 11th day after inoculation.

A. Strain $C^+ H^-$ "strong" CPE.
B. Strain $C^- H^+$ "slight" CPE.
C. Uninoculated culture.


6A
6B
6C
6D



Gotlieb-Stematsky and Leventon.

centage of animals developing tumours as a result of inoculation with the $C^+ H^-$ strain was lower at the various dilution levels than with the $C^- H^+$ strain. Also a great difference in the intensity and spread of tumours and haemorrhages was noted between the 2 strains, the most conspicuous being a high percentage of haemorrhages in the livers and lungs of animals inoculated with the $C^- H^+$ strain.

DISCUSSION

The question whether mouse leukaemia and/or virus-induced mouse tumours are of a single or multiple viral aetiology has been raised in the past by various investigators engaged in experimental work in this field (Graffi, Bielka and Fey, 1956 ; Gross, 1958 ; Friend, 1959 ; Sachs *et al.*, 1959 ; Eddy and Stewart, 1959). The findings that the virus capable of inducing mouse tumours can be grown in cell cultures (Stewart *et al.*, 1957 ; Eddy *et al.*, 1958), opened the possibility of using the plaque technique in an attempt to isolate pure virus lines for further study. By using the plaque technique, we were able to isolate at least 2 distinct plaque variants from the parent SE polyoma virus. In addition to the difference in plaque size, virus lines derived from these 2 distinct plaques were shown to differ markedly in other characteristics like CPE and haemagglutinating activity, and their tumour inducing capacity. Since no detailed histological analysis was attempted in further differentiation of the tumour types caused by these strains and their blood and bone-marrow pictures, their identity and relation to the other strains causing mouse leukaemia cannot be determined. It was evident, however, that these 2 strains differ in their tumour inducing capacity and organ-preference in mice and hamsters. The widespread occurrence of SE polyoma virus in normal mice (Rowe, Hartley, Brodsky, Huebner and Law, 1958 ; Rowe, Hartley, Estes and Huebner, 1959) raises the possibility that one of these strains originated from our mouse cell cultures. This cannot be ruled out altogether since we succeeded by repeated subcultures to isolate a virus closely related to SE polyoma from our mouse colony. In addition, it was observed that our uninoculated mice have varying HI titres against SE polyoma virus. Against this assumption stands out the fact that we were able to repeat several times the isolation from plaques of these 2 virus lines. The characteristics of the 2 virus strains, $C^- H^+$ and $C^+ H^-$, were maintained after inoculation in animals. It was also possible to isolate strains of varying CPE and HA characteristics directly from animals inoculated with the parent SE polyoma virus without the plate method.

The most important feature which deserves special mention seems to be the differences observed in the tumour-inducing capacity of the 2 variants, $C^- H^+$, and $C^+ H^-$. The difference in their capacity to induce tumours in both mice and hamsters varied significantly. This character seemed to be linked with the haemagglutinating capacity of the strain ; high HA titres were always correlated with a high percentage of animals with tumours, whereas low HA titres—with few or no tumours.

Data obtained from the growth curves of the 2 strains in the fluids and inside the cells may point to a different mechanism of cell destruction and virus release. The $C^- H^+$ strain is presumably released continuously from the cells and therefore the virus content, as measured by HA, is higher in the fluid than in the cells, and little cell destruction occurs. The behaviour of the $C^+ H^-$ strain is different in

this respect. Virus seems to accumulate early in the cells causing cell destruction during cell burst.

Since the less cytopathogenic strain is also the one that induces a higher percentage of tumours in animals, it remains to be proved whether its behaviour at the cellular level in tissue culture can be translated into what occurs *in vivo*; especially in view of our experimental findings that cell destruction by this strain *in vitro* could be kept at a minimum for a long period of time if changes of medium were carried out at frequent intervals.

The role of bicarbonate concentration and its effects on the growth of the 2 strains is of interest in view of the findings obtained; its role in synthesis of protein and RNA has been recently pointed out by Chang (1959). Whether bicarbonate concentrations have an influence on the tumour inducing capacity of these strains remains to be investigated.

SUMMARY

The isolation of 2 strains of virus from the parent SE polyoma virus and the description of their characteristics is reported. These strains, designated C^- H^+ and C^+ H^-, were isolated from plaques of different size.

The C^- H^+ strain was isolated from a large plaque, 3–4 mm. in size, and was characterized by a limited CPE, high HA titre and a high tumour and haemorrhage inducing capacity in new-born mice and hamsters. The C^+ H^- strain came from a small plaque, diameter 1–1·5 mm., was highly cytopathogenic, gave low HA titres and was limited in its tumour-inducing activity.

The growth characteristics of the 2 virus strains as compared with the parent strain are presented. It was found that the pH of the medium and especially the bicarbonate concentration have a marked influence on the various characteristics of the 2 strains. The relationship between the *in vitro* and *in vivo* behaviour of the strains is discussed.

We wish to thank Dr. N. Goldblum for his valuable help and encouragement throughout this investigation, and to the Misses M. Hermann, E. Stambulka and Y. Shaya for their able technical assistance.

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THE DEVELOPMENT OF AN ANASTOMOTIC CIRCULATION TO TRANSPLANTED TISSUE

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ATHEROMA and accompanying thrombosis in certain vessels such as the coronaries of the heart and the cerebral vessels are so serious and occur so frequently that great efforts are now being made to understand the underlying processes, in the hope that it may be possible at least to slow down their evolution, if not to prevent them altogether. Efforts have also been directed to the possibility of bringing, by surgical means, additional supplies of blood to a heart rendered ischaemic by the narrowing or occlusion of its coronary vessels. One such method has been the grafting of tissues on to the surface of the heart in the hope that vascular connections will develop between extraneous vessels and those of the coronary system. A variety of vascular tissues has been tried and in most cases vascular anastomosis has been demonstrated by injecting the vessels of the heart through those of the graft or *vice versa* (Beck and Tichy, 1935; O'Shaughnessy, 1936; Lezius, 1937; Heinbecker and Barton, 1939; Burchell, 1940; Thompson and Raisbeck, 1942; Beck, 1943; Scola and Stoesser, 1944; Vineberg, 1946 and 1949; Carter, Gall and Wadsworth, 1949; Riemann, Cowley and Raby, 1950). No photographs illustrating the connections have been published and it would seem likely that although these may be numerous they are probably very small. It has yet to be shown that the direction of blood flow in such anastomoses is towards the heart. von Wedel, Greenwich, Lord, Neumann and Hinton (1955) could find no evidence that blood was flowing towards the heart in experiments designed to show the direction of blood flow in their pedicle skin grafts.

Nevertheless it was felt that this question should be further investigated and it was thought that it would be helpful to use a simpler system than the heart and coronary vessels to gain some insight into the formation of vascular anastomoses between one tissue and another. The preparation described by Florey and Harding (1935), which consisted of a length of duodenum that had been transplanted into the subcutaneous tissue of a rabbit, seemed suitable for this purpose. These workers had found that when the mesenteric blood supply was subsequently severed the transplanted duodenum not only survived but continued to secrete mucus. It seemed probable that vessels from the subcutaneous tissue were providing a new circulation.

This paper describes the kind of vascular connections which were found to have formed in transplants of this type.

EXPERIMENTAL

The duodenum was transplanted in 100 fully grown rabbits of mixed breed. The technique was similar to that used by Florey and Harding (1935). For operative procedures anaesthesia was induced with intravenous nembutal and deepened with ether. The duodenum was located through an upper midline abdominal incision, and a portion 3–4 cm. long, still attached

to the mesentery, cut free. Duodenal continuity was restored by end to end anastomosis carried out over a polythene tube to prevent stenosis. The upper end of the isolated portion was insewn, and the sac so formed was delivered through a stab incision 5 cm. from the midline. The closed end of the sac was drawn into a subcutaneous pouch, while its mouth was sutured to the edges of the stab wound (Fig. 1). The abdomens of some of the animals were reopened 6 weeks later and the pedicle of mesentery, which was running to the transplanted duodenum, was divided between ligatures.

The animals were killed at varying intervals after the initial transplant and the vascular connections were studied in one of two ways. (1) The arteries were injected with neoprene latex, by way of a cannula placed in the ascending aorta, and a dissection was subsequently carried out. (2) The vessels were filled with an India ink and gelatine mixture and serial sections cut through the transplant. For the most part thick sections (250 μ) were cut, the tissue being first embedded in nitrocellulose according to the technique of Chesterman and Leach (personal communication). The sections were stained lightly with haematoxylin and eosin by immersing them successively in distilled water for 15 min., 1 per cent acetic acid for

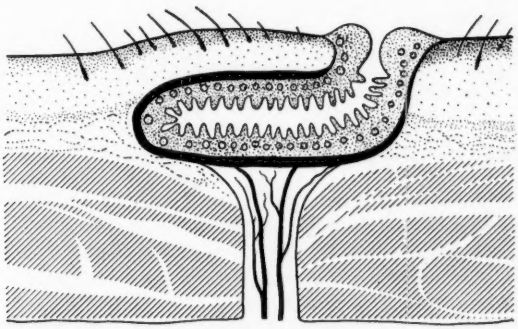


FIG. 1.—The position in the anterior abdominal wall occupied by the duodenal transplants.

15 min., 10 per cent Harris's haematoxylin for 1 hr., 1 per cent hydrochloric acid in 70 per cent alcohol for 4 min., tap water for several hours, 0·005 per cent eosin for 20 min., 70 per cent alcohol for 20 min., equal parts of xylol, alcohol and chloroform for 40 min. Handling of the sections was facilitated by threading them on to long safety pins, a glass bead intervening between each section.

RESULTS

The transplants were well tolerated by most of the rabbits. Some inflammation of the nearby skin occurred, but was controlled with applications of Baltimore paste (Martindale, 1952). Division of the mesenteric pedicle usually produced no change in the appearance of the fistula or of its secretion, although in a few cases a sticky plug formed in the mouth of the fistula and occasionally a small infarct was visible.

Histological observations

Sections of transplants which had been in place for only a few days showed inflammatory changes both in the tissues of the duodenum and in the surrounding subcutaneous tissue, where a considerable number of extravasated erythrocytes were present. During the first week a cellular zone, containing numerous small blood vessels, appeared around the transplant and it became impossible to deter-

mine exactly the boundary between duodenal and subcutaneous tissue. The serosa was at no time obvious. Later this cellular zone was replaced by fibrous tissue, and abnormally large blood vessels were to be seen in the neighbourhood.

The fistulous pouch itself looked like normal duodenum although there was a tendency for the villi to be shorter than usual, and in some cases adjacent villi were fused together. In a few cases areas were found with apparently no villi at all, but in others, even after a year, the appearances were quite normal.

Two rabbits were killed 1 day and 3 days after division of the mesenteric pedicles to their fistulae. Formol saline was gently run into the mouth of each fistula to ensure rapid fixation. There was no sign of necrosis although the cells were rather distended and diffuse patches of eosinophilic material were visible in the submucosa.

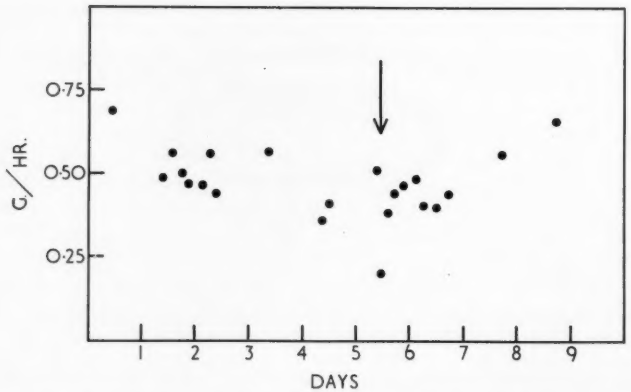


FIG. 2.—The rate of secretion from a duodenal transplant measured on successive days. The arrow indicates the time at which the mesenteric pedicle of the transplant was divided.

Rate of secretion

This was measured by applying an absorbent pad to the mouth of the fistula and noting the increase in weight of the pad in a given time interval. The rate of secretion was followed in three transplants before and after division of their mesenteric pedicles. This operation caused only a transient fall in the rate of secretion in two cases, one of which is illustrated in Fig. 2, while in the third the rate was at first unchanged but later became greater than before the pedicle was cut.

Dissection of injected specimens

Dissection of specimens which had been injected with neoprene revealed many abnormally large vessels in the subcutaneous tissue around the fistula. In one rabbit the right internal mammary artery, which supplied these vessels, was twice as large as the internal mammary artery on the left side and was, in fact, almost as large as the common carotid artery. It was also tortuous (Fig. 3).

Connections between vessels of the transplant and vessels of the surrounding subcutaneous tissue were found in most dissections. In some cases only fine

TABLE I.—*Sizes of Connections to Fistulae with Serosa Intact*

Days since transplantation	Days since pedicle division	Arterial connections (μ)	Venous connections (μ)
	Pedicle intact		
21	—	90, 90	—
48	—	—	—
88	—	—	—
125	—	—	220, 120
211	—	100	150
352	—	40, 40, 40	200
356	—	—	140, 140, 140
425	—	—	250, 100
	Pedicle divided		
65	16	70, 70, 70	—
111	69	80, 80, 50	300, 80
151	108	70	150, 150, 100, 70
169	125	70, 30	150, 50
172	126	100, 100	—
229	185	100	200, 200
253	208	100, 30, 30	80
397	345	100, 70	—
415	370	120	—
416	371	20, 20	80

vessels were present, but in others quite large ones, both arterial and venous, had formed (Fig. 4, 5 and 6). The kinds of connection which were found in one group of rabbits are listed in Table I. In about half of these the mesenteric pedicle had been divided at some time before the injection, while in the remainder the pedicle was intact up to a few minutes before making the injection. It will be seen that fairly large venous connections were found in all transplants which had been in place for more than a few weeks, whereas arterial connections were on the whole larger in those transplants whose pedicles had been divided some weeks before the injection. In a smaller group of rabbits the serosa was stripped off the duodenal pouch before it was implanted in the subcutaneous tissue. The connections which formed in these fistulae (Table II) were larger than in those in which the serosa had been left intact (Table I). However, none of the connections compared in size with the original pedicle artery which was about 1 mm. in diameter.

TABLE II.—*Sizes of Connections to Fistulae without Serosa*

Days since transplantation	Days since pedicle division	Arterial connections (μ)	Venous connections (μ)
	Pedicle intact		
63	—	—	150
81	—	—	100, 100, 100
199	—	80, 50, 50	80
290	—	100	—
	Pedicle divided		
146	62	150, 100	200, 200
206	124	150, 150, 50	150, 100, 60
269	186	120, 50, 50	250, 200, 100
458	374	200, 120	350

From the course of a particular connection it was sometimes possible to infer the direction in which the blood flowed. For instance, in one case an arterial connection of 200 μ arose from an artery in the subcutaneous tissue and broke up into a number of tapering branches as it approached the fistula. In another the appearance of the connections suggested that the flow was away from the fistula.

Microscopic examination of injected specimens

Anastomoses capable of conveying India ink particles were present within a few days of transplantation (Fig. 7). Connections were found in many of the specimens examined. In most cases these ran obliquely and had to be traced through a number of consecutive sections, but in some instances they lay within one or two sections and could be photographed (Fig. 8, 9 and 10). The walls of arterial connections contained smooth muscle cells which, except in the largest connections, formed a single, circularly arranged layer. The walls of venous connections were thinner and consisted of little more than endothelium. Some of the vessels leaving the duodenum tapered and branched when traced out into the subcutaneous tissue. In most cases, however, they joined up with larger vessels. Venous connections joined veins in the transplant to veins in the subcutaneous tissue; arterial connections joined arteries to arteries.

Vascular adhesions of the heart

A series of experiments was carried out on pigs to test the effectiveness of cellophane as an agent to bring about gradual coronary occlusion. Some narrowing of the coronary arteries occurred but in no case was there total occlusion. It was noticed, however, that numerous vascular adhesions had formed between the heart and the adjacent pericardium and chest wall. Most of the vessels were minute but a few larger ones were seen, one of which is illustrated in Fig. 11.

EXPLANATION OF PLATES

FIG. 3.—The inner aspect of part of the anterior chest wall of a rabbit which had a duodenal transplant in its right anterior abdominal wall. Approx. $\frac{2}{3}$ of full size.

FIG. 4.—A drawing of a dissection of a transplant. The muscular layers of the abdominal wall have been removed and the length of duodenum so exposed has been lifted up to reveal an arterial connection which is running from a small artery in the subcutaneous tissue to one of the original arteries in the duodenum. ×3.

FIG. 5.—A drawing of a dissection showing a small arterial connection (C) linking an artery in the subcutaneous tissue with a vessel in the duodenum. ×3.

FIG. 6.—A drawing of a dissection showing a small artery in the subcutaneous tissue giving off several branches to a duodenal transplant. ×3.

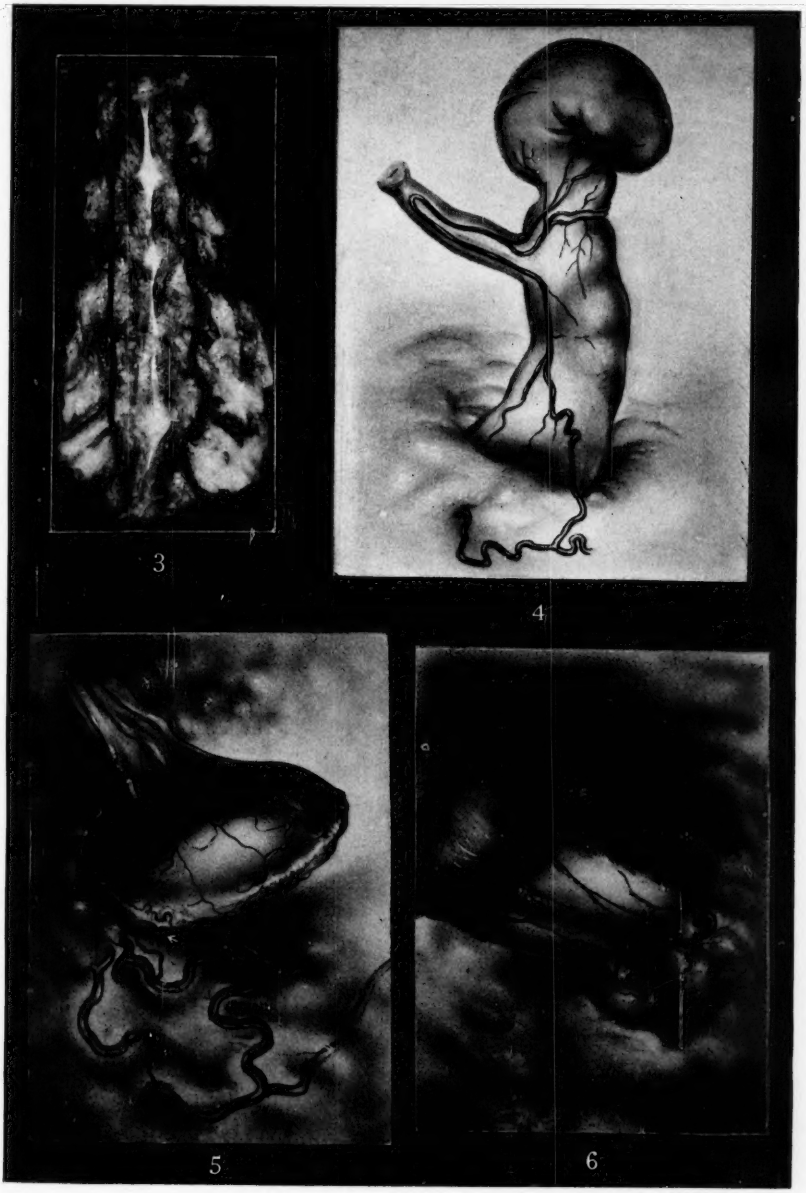
FIG. 7.—A section through a duodenal transplant. The rabbit was killed 4 days after transplantation and its superior mesenteric artery injected with India ink. Particles of ink can seen in some of the vessels in the subcutaneous tissue at arrows. ×16.

FIG. 8.—A section through a duodenal transplant. The vessels have been filled with India ink. A vein leaves the transplant and runs out into the subcutaneous tissue at the arrow. ×16.

FIG. 9.—A section through a duodenal transplant. A small artery can be seen at the arrow entering the duodenum from the overlying subcutaneous tissue. ×16.

FIG. 10.—A number of fine vessels can be seen traversing the gap which separates the duodenum from the subcutaneous tissue. ×16.

FIG. 11.—A photograph of a dissection showing a small artery (diameter 250 μ) leaving a coronary artery (CA) and breaking up into a number of branches which enter the chest wall (CW). The arteries were injected with neoprene latex.



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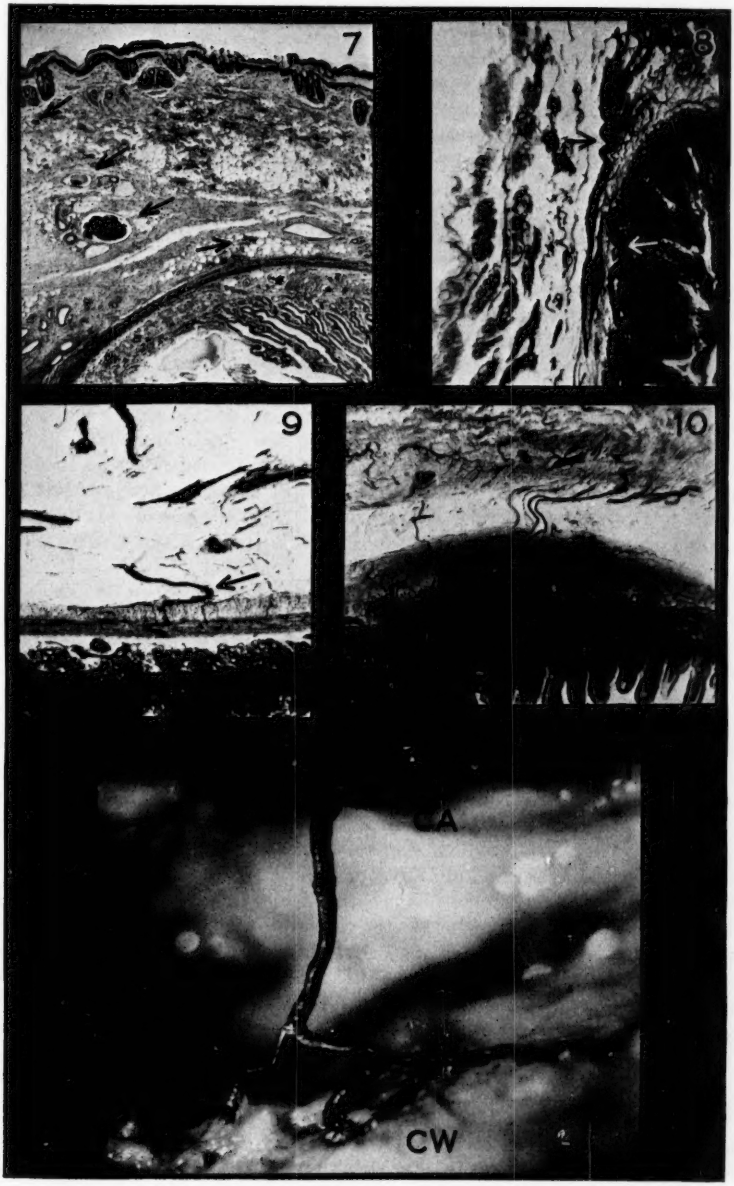

North, Sanders and Florey.

DISCUSSION

It is clear from this study that vascular connections between apposed tissues do form. These were no mere capillaries but vessels capable of nourishing a length of transplanted duodenum. Just how they formed is a matter for surmise, but it is probable that the initial union took place between capillaries. There was a period, a few days after transplantation, when India ink but not neoprene could be made to traverse the anastomoses. Presumably capillary buds had grown out from both the duodenal and subcutaneous tissue into the damaged zone immediately surrounding the transplant, and had united in much the same way that capillaries have been seen to do in an ear chamber (Clark and Clark, 1939 and 1943). From such a capillary network small arteries and veins have been seen to form (Clark and Clark, 1939 and 1943) and it would seem likely that this is what took place in our duodenal transplants. It was interesting to observe that the anastomotic circulation made use of pre-existing vessels in the duodenum. Studies which have been made on the development of collateral circulation suggest that haemodynamic factors are responsible for the growth of collateral vessels (Nothnagel, 1889; Thoma, 1896; Longland, 1953; North and Sanders, 1958). When one of two competing channels is severed it has been found that enlargement of the undivided vessel takes place (North and Sanders, 1958). It was noticeable in the present study that it was in the transplants, the mesenteric pedicles of which had been divided, that the largest arterial connections were found.

In the case of grafts applied to the surface of the heart, there is ample evidence that anastomosis at a capillary or small vessel level occurs (Beck and Tichy, 1935; O'Shaughnessy, 1936; Lezius, 1937; Heinbecker and Barton, 1939; Burchell, 1940; Thompson and Raisbeck, 1942; Beck, 1943; Scola and Stoesser, 1944; Vineberg, 1946, 1949; Carter *et al.*, 1949; Baronofsky, Sprafka and Noble, 1954; Bloomer, Stern and Liebow, 1954; von Wedel *et al.*, 1955; Elzenbaum, 1955). The fact that no large connections have been demonstrated suggests that it is the factors concerned with the enlargement of these connections which are not favourable. We have found quite a small artery growing out from the coronary system to supply adherent tissue, and Kline, Stern, Bloomer and Liebow (1956) have demonstrated large connections between the coronary and bronchial arteries following ligation of a pulmonary artery, but so far no one has shown connections of any size bringing blood to the heart. Even when the coronary arteries have been gradually occluded no significant extravascular anastomoses have formed (Burchell, 1940) although large intercoronary anastomoses have developed in these circumstances (Mautz and Beck, 1937; Zoll, Wessler and Schlesinger, 1951; Beck and Leighninger, 1954; Baroldi, Mantero and Scomazzoni, 1956). Perhaps the peculiar pressures prevailing in the coronary arteries account for this.

SUMMARY

Vascular connections were formed between the vessels of a transplanted length of duodenum and those of the surrounding subcutaneous tissue.

Some of these connections were arterial, some venous. The arterial connections ran between arteries of the duodenum and arteries of the subcutaneous tissue. The venous connections linked veins to veins.

The earliest occasion on which connections were demonstrated was four days after transplantation.

Reasonably large venous (150 μ) and arterial (100 μ) connections were present in most of the fistulae whose pedicles had been divided and in some of the fistulae whose pedicles were left intact. The largest arterial connection found was 200 μ in diameter. The largest venous connection was 350 μ. Connections were, on the whole, larger in those fistulae whose serosa had been stripped off before implantation.

Most of the fistulae were adequately nourished after their pedicles had been divided and continued to secrete typical rabbit duodenal juice. Yet in some cases the diameter of the largest arterial connection was less than one-sixth of the diameter of the original pedicle artery.

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THE EFFECT OF HYPOXIA ON LIVER SECRETION STUDIED BY INTRAVITAL FLUORESCENCE MICROSCOPY

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INTRAVITAL fluorescence microscopy was introduced in 1929 by Ellinger and Hirt (1929*a*, 1930*b*). They developed the method so that direct observations could be made of organs of living animals under the microscope. Because of the size and opacity of most organs, transmitted illumination could not be used, and it was necessary to employ incident light. In the ordinary way, incident light illumination gave a poor image of the underlying parenchyma because it was either reflected from the surface or absorbed by the superficial layers. It is true that Vonwiller (1927) had attempted to improve the image by introducing artificial reflectors into the tissues, but these were of little practical value. What was really required was a source of light emanating from the object, with no interference by reflection. This requirement was met by exciting fluorescence in the tissues with incident ultra-violet light. A filter in the eyepiece would prevent any ultra-violet light from reaching the eye, but light of longer wave-length formed in the tissues by the phenomenon of fluorescence was able to pass the filter and form the image. Most tissues were themselves fluorescent under incident ultra-violet illumination but fluorescence was greatly enhanced by the use of fluorescent dyes.

Thiel in 1926 obtained a brilliant picture of fluorescence in the capillaries and lymphatics of the human eye. He gave his patients a large dose of fluorescein by mouth and then viewed the eye with an ultra-violet slit-lamp. It was Ellinger and Hirt, however, who first examined internal organs by this method, and they were able to demonstrate secretory activity in the liver and kidney. Fluorescein was actively secreted by the liver into the bile and by the kidney into the urine, and its passage through the parenchymal cells of these organs was observed (Ellinger and Hirt, 1929*b* and *c* ; 1930*a* and *b* ; 1931). Since then, Franke and Sylla (1933), Grafflin (1947), Kulpe (1955*a* and *b* ; 1956) and others have investigated the secretion of a number of fluorescent dyes by the liver of amphibians, and the effects of chloroform, carbon tetrachloride and other liver poisons on this secretion.

Grafflin and Bagley (1952) applied this experiment to a number of species of mammals ranging from the mouse to the monkey, but it was due to Hanzon (1952) that intravital fluorescence microscopy could be used for quantitative studies on secretory function in mammalian liver. Hanzon chose the rat as his experimental animal ; it was an animal of convenient size, and many had used it in the past for studying liver damage. By measuring the amount and contents of the bile, he was able to show that the liver secreted sodium fluorescein in much the same way as it secreted sodium bilirubin.

This paper outlines the technique of intravital fluorescence microscopy applied

to the secretion of sodium fluorescein by the liver of the rat. It describes the pattern of secretion in the normal animal and how this is altered by varying degrees of hypoxia.

MATERIALS AND METHODS

Subjects and anaesthesia.—Male Wistar albino rats whose weight varied from 150 to 400 g. were fed on the standard 41B diet with free access to food and water. Twenty-four hours before they were used, food was withdrawn and they were allowed water only. They were anaesthetized with 2 per cent Numal-Roche (diethylamino allyl isopropyl barbituric acid) by intraperitoneal injection. It gave deep narcosis for 3 hr. in a dose of 0·5 ml. per 100 g. body weight. If the experiment was prolonged beyond this time, a further dose of half this amount was injected subcutaneously.

Preparation.—When the animal was asleep, it was secured to a perspex tray which could be fitted over the stage of the microscope. A mid-line incision was made in the skin of the neck, the salivary glands and strap muscles were divided and a tracheotomy was performed. A polythene tube, 5 cm. long and 0·2 cm. in diameter, was tied into the trachea and, if necessary, this could be sucked out by a needle attached to a 20 ml. syringe. The right jugular vein was gently dissected and the distal end ligatured. A polythene cannula, rinsed in a little heparin solution, was inserted just proximal to this ligature through a V-shaped cut in the wall of the vein made with iridectomy scissors. The cannula was tied in position and a Gordh needle was inserted into its open end. During the cannulation, the foot of the tray was raised about 1 cm. and a bulldog clip was put on the proximal part of the vein to prevent air embolism. The Gordh needle was secured in a block of plasticine which was stuck to the tray beside the animal's head.

The abdomen was then opened by a transverse incision just below the level of the costal margin. The xiphisternum was excised and the falciform ligament of the liver divided. A specially shaped perspex retractor was placed between the liver and the diaphragm and was clamped in position. The lower edge of the lobe of the liver to be examined (usually the large left lateral lobe) was lifted up by a glass rod bent into the shape of a golf club at one end and securely clamped at the other. The ventral surface of the lobe was allowed to adhere by surface tension to the underside of a 3 × 2 cm. microscopic cover glass fixed to a perspex rod which was secured in position by yet a third clamp. All the clamps were fixed to the edge of the tray which held the animal. With care, the lobe could be kept still despite respiratory movement. The surface of the liver was kept at 38·0 ± 0·3° by a constant stream of warm physiological saline prepared as follows: Sodium chloride 8·0 g., potassium chloride 0·2 g., calcium chloride 0·2 g., magnesium chloride 0·1 g., sodium bicarbonate 0·35 g., glucose 1·0 g., distilled water to 1 litre. This solution issued from a glass pipette just above the coverslip at the rate of 20–25 ml. per min. The saline from a glass aspirator bottle passed through coiled glass tubing immersed in a tank of water kept at 39·0 ± 0·1° by a thermostatically controlled heating unit. The temperature of the surface of the liver was measured with a copper-constantin thermocouple placed beneath the coverslip. The tray was fitted over the microscope stage and the objective focussed on the surface of the liver through the cover glass.

Microscope, light source and photography.—The Leitz "Panphot" microscope used for these experiments was fitted with a special lamp housing containing both a low voltage tungsten filament lamp and a Philips ME/D 150-watt discharge lamp. This lamp has a discharge tube of thick quartz which contains mercury vaporized in argon. It operates at a very high pressure so there is a continuous spectrum as well as the mercury lines. The tungsten electrodes are separated by a gap of only 0·2 cm. giving a compact source with a brightness more than twice that of a 15 amp. carbon arc. The light was passed through a 4 mm. BG12 filter which admitted visible violet light between 390 and 440mμ wave-length. An orange filter, 2·5 mm. OG1, was placed in the eyepiece to cut out all light of wave-length shorter than 510mμ (Gottschewski, 1954). The objective was a Leitz water immersion "Ultropak" ×22, which provided by means of annular condensers, powerful incident light illumination. So there should be as little loss of light as possible, the mirrors between the light source and the objective were surface aluminized. Photographs were taken with an "Exacta" 35 mm. reflex camera and a ×8 eyepiece. Using a fast film (Ilford H.P.3), the exposure required to photograph liver containing fluorescein was 20 sec. and a photograph of autofluorescence in

the liver needed three times this exposure. Care was taken not to expose the liver to the intense violet light longer than was necessary to make observations and take photographs.

The sodium fluorescein employed was the brand of "Uranin" supplied by George T. Gurr, Ltd. A 2 per cent solution in saline was prepared and the standard dose given in each experiment was 0·1 ml. per 100 g. body weight. This was injected with a tuberculin syringe and needle, through the rubber diaphragm of the Gordh needle in the jugular vein, while the liver was being observed under the microscope.

Production of hypoxia.—In a few initial experiments, the liver was rendered hypoxic by ligature of the hepatic artery. When this was done too close to the coeliac axis, the liver received arterial blood from collaterals and behaved in a completely normal fashion. When the hepatic artery was tied close to the liver, there was naked eye pallor of the organ. The liver cells took up the fluorescein but it was not secreted into the canaliculi. This way of reducing the oxygen supply to the liver was unsatisfactory, so animals were made instead to breathe a gas mixture which contained known low concentrations of oxygen. Oxygen and nitrogen from cylinders were mixed in the flowmeter chamber of an anaesthetics apparatus. The percentage of oxygen in the mixture was continuously measured by an instrument devised by Pauling, Wood and Sturdivant (1946) which depends on the magnetic susceptibility of oxygen being very much greater than that of any other common gas. The mixture was led through a tube ending in a T-piece, the side arm of which was connected to the endotracheal tube in the animal, and the end of which was open to the air. It was important to ensure that sufficient gas mixture passed through the tube so that the mixture inhaled was uncontaminated by expired gas which would have increased the hypoxia to a degree which could not be measured. With the system described, rebreathing cannot occur if the fresh gas flow exceeds the peak inspiratory flow rate of the rat. If it is assumed that the respiratory excursion of the animal resembles simple harmonic motion, the peak volume flow required is $\pi \times$ *minute volume*. The tidal air was measured in 11 rats by observing the movement of a soap film in the barrel of a 2 ml. graduated hypodermic syringe connected to the endotracheal tube. It ranged from 0·9 to 1·3 ml. with a mean of 1·07 ml. The average number of respirations per minute was 70 but sometimes this went up to 140, especially if the rat was given a halogen containing anaesthetic. With a respiratory rate of 140 per min. and a tidal air of 1·3 ml., $\pi \times$ *minute volume* is 573 ml. To be certain that no rebreathing could occur, at least 700 ml. of gas per min. were delivered throughout all the experiments.

RESULTS

Before injecting the dye, autofluorescence of the liver was observed. With *ultra-violet* light, (using a 4 mm. UG1 filter, with a 2·5 mm. Euphos eyepiece filter), the cytoplasm of the liver cells showed a blue fluorescence, the nuclei looking dark in contrast. The sinusoids were dark also and the red cells appeared as moving shadows. In a number of livers, the Kupffer cells contained granules which showed a bright yellow fluorescence that tended to fade in the ultra-violet light (Fig. 1). With *violet* light, (using a 4 mm. BG12 filter, with a 2·5 mm. OG1 eyepiece filter), the autofluorescence of the liver cells was greenish and difficult to distinguish from fluorescein fluorescence except that it was very much less intense. The Kupffer cell granules were orange.

A few seconds after the intravenous injection of the dye, a brilliant yellow-green fluorescence appeared in the parts of the sinusoids adjacent to the portal areas. The portal tracts themselves were not seen, as only rarely do they appear on the surface. The fluorescence rapidly spread throughout the sinusoids and into the "centrilobular" veins, which unlike portal tracts always appear beneath the capsule. The endothelial lining of the sinusoids appeared to be stained by the fluorescein (Fig. 2), but very soon the liver cells took up the dye and became strongly fluorescent (Fig. 3). Perhaps half a min. later, the bile canaliculi became visible as fine branching lines of brilliant fluorescence running through the centres of the columns of liver cells (Fig. 4). In the continuous sheet of liver cells beneath

the capsule which are not part of the lobular system and are not in columns, the canaliculi formed a network with branches communicating with those canaliculi in the columns beneath (Fig. 5). The canalicular fluorescence continued to increase and became even more striking as the liver cells lost their fluorescence (Fig. 6). Fluorescein was completely eliminated from the liver cell in about 45 min. but persisted in the canaliculi for 2–3 hr. When the surface of the liver was insufficiently irrigated with warm saline, its temperature dropped and the sequence of events described was slowed down. This fact was made use of to obtain the series of photographs in Fig. 2–5. With rigid temperature control at 38·0 ± 0·3°, the time was recorded for the liver cell to take up the fluorescent dye and for the dye first to appear in the bile canaliculi. This experiment was performed on 6 freshly prepared animals breathing room air and the results are given in Table I.

TABLE I.—*Time in sec. for Fluorescein Secretion under Normal Conditions*

Expt. No.	Time from sinusoid to liver cell	Time from sinusoid to bile canaliculus
1	9	61
2	17	46
3	16	44
4	13	55
5	7	27
6	5	41
Mean	11·2 (SD = 4·9)	45·7 (SD = 15·3)

The average time for fluorescein to be taken up by the liver cell after its appearance in the sinusoid was 11·2 sec. (SD = 4·9), and for it to be seen in the bile canaliculi after its appearance in the sinusoid 45·7 sec. (SD = 15·3).

To study the effects of hypoxia, a standard experiment was devised. The intraperitoneal injection of anaesthetic was given and the animal prepared in the manner described, and placed in position under the microscope. The period of hypoxia was started 1 hr. after the induction of the anaesthesia. The gas mixture was maintained constant within ±0·3 per cent for any given experiment at 6·0, 7·0, 8·0, 10·0 or 11·0 per cent oxygen in nitrogen. The hypoxia was continued for an hour before the standard dose of fluorescein was injected. The secretion was timed and the liver observed over the hour which followed the injection. After 2 hr. hypoxia, the rat was again permitted to breathe room air. An hour later, a second dose of fluorescein was injected and the secretion was again timed, this time with the animal breathing air. The results of these experiments are given in Table II. With hypoxia, the average time for fluorescein to be taken up by the liver cell after its appearance in the sinusoid was 13·4 sec. (SD = 4·0), and for it to be seen in the bile canaliculi after its appearance in the sinusoid 86·3 sec. (SD = 24·1). The times for the second dose after breathing room air for 1 hr. were 8·2 sec. (SD = 2·4) and 44·9 sec. (SD = 8·3) respectively. In Table III, the 95 per cent confidence limits of the mean times in both Tables I and II are given for secretion from sinusoid to bile canaliculus. There is a considerable gap between the upper limits under conditions of air breathing and the lower limit under hypoxic conditions. This means that the increase in secretion time with hypoxia is highly significant.

There was no correlation between the time of secretion and the degree of

TABLE II.—*Time in sec. for Fluorescein Secretion During and after Hypoxia*

Expt. No.	Percentage oxygen	After 1 hr. hypoxia: Time from sinusoid to liver cell	After 1 hr. hypoxia: Time from sinusoid to bile canaliculus	2 hr. later after 1 hr. air: Time from sinusoid to liver cell	2 hr. later after 1 hr. air: Time from sinusoid to bile canaliculus
1	11	27	91	—	—
2	11	11	46	—	—
3	11	12	80	11	38
4	10	9	67	10	45
5	11	5	101	6	48
6	10	10	50	7	41
7	10	9	91	8	41
8	10	11	81	7	41
9	8	22	85	6	31
10	8	19	111	5	51
11	8	11	74	7	46
12	8	14	105	6	53
13	7	15	67	6	30
14	7	20	149	14	64
15	7	9	88	9	55
16	7	14	71	8	41
17	6	12	69	10	47
18	6	14	103	9	47
19	6	10	111	11	45
Mean		13·4 (SD = 4·0)	86·3 (SD = 24·1)	8·2 (SD = 2·4)	44·9 (SD = 8·3)

hypoxia, but as the hypoxia became more severe, the microscopical appearances changed greatly. In all the hypoxic animals, it was noticed that the dye was taken up by the liver cells more quickly at the periphery of the lobule than at the centre. Of the 8 animals breathing 11 and 10 per cent oxygen, 4 showed no other abnormality, and on reinjection under conditions of air breathing, no difference between central and peripheral cells was seen. In the other 4 animals, about 20 min. after the fluorescein injection, a number of vacuoles of bright fluorescence were seen in the liver cells at the centres of the lobules. These vacuoles represented slight damage to the liver cells in this zone and were present on reinjection with fluorescein after 1 hr. of air breathing. With 8 per cent oxygen, centrilobular damage was constant. No fluorescent canaliculi appeared in the cells at the centres of the lobules, and after about 15 min., the cells at the boundary between functioning periphery and inactive centre were filled with vacuoles. It was as if there was damage to the canalicular wall and the fluorescent substance flowing along normal canaliculi leaked out when it came to the damaged boundary zone and produced the brightly fluorescent vacuoles (Fig. 7). On reinjection, the damaged centres failed to secrete, and the boundary zone vacuoles again appeared. The picture at 7 per cent oxygen was similar, the boundary zone vacuoles appearing after

TABLE III.—*Ninety-five per cent Confidence Limits of Mean Times for Secretion from Sinusoid to Bile Canaliculus*

	Table I	Table II	
	Air	Hypoxia	Air
Number of expts.	6	19	17
Mean time in sec.	45·7	86·3	44·9
Standard deviation	15·3	24·1	8·3
Standard error of mean	6·2	5·5	2·0
Ninety-five per cent confidence limits of mean	33·3–58·1	75·3–97·3	40·9–48·9

5 min. At 6 per cent oxygen, it was noticed that autofluorescence of the centres was poor before the fluorescein was injected. Only a small rim of liver cells surrounding the portal tracts functioned at all. Boundary zone vacuoles soon appeared with a large, non-functioning central zone occupying about three-quarters of the total liver surface. After an hour of air breathing a little recovery took place, and the picture on reinjection was not so severe, but nevertheless more than half the lobule was damaged. No experiments could be performed at less than 6 per cent oxygen for such mixtures were quickly lethal.

Blocks of liver from these animals were taken, and after formol fixation and paraffin embedding, sections were cut and stained with haematoxylin and eosin. A remarkable degree of autolysis of the damaged liver cells was often seen, with brick-red cytoplasm and pyknotic nuclei. This appearance, confined of course to the central zones, was most marked near the surface of the organ (Fig. 8).

DISCUSSION

The removal of fluorescein from the blood stream by the liver cells, and its subsequent secretion into the bile canaliculi, are two processes which can be directly observed by the method described. Hanzon (1952) showed that the liver was able to concentrate sodium fluorescein about 500 times. He found that liver cells dealt with this substance and with sodium bilirubin similarly when they were injected into the blood stream, and their concentrations in the blood and bile subsequently measured. The only important difference was that fluorescein caused an increase in bile volume whereas bilirubin did not. The similarity is sufficiently close for one to assume that the same mechanisms are used for the secretion of fluorescein as are required for the normal elimination of bilirubin in the bile. Another acid dye-stuff which has proved most valuable as a test substance for liver function is bromsulphthalein which is probably also secreted in the same way. Brauer and Pesotti (1949) found that this could be concentrated up to 900 times by the liver of the rat.

EXPLANATION OF PLATES

FIG. 1.—Autofluorescence of normal liver in the living animal. The columns of liver cells are faintly fluorescent with Kupffer-cell granules showing brilliantly by contrast. ×230.

FIG. 2.—The normal liver just after the sodium fluorescein has entered the sinusoids. The dye is present in the sinusoids and has stained the endothelial lining. ×230.

FIG. 3.—The same field a short time later. Fluorescein is no longer in the sinusoids but the columns of liver cells are now brightly fluorescent. ×230.

FIG. 4.—Later still, the dye has begun to be concentrated in the bile canaliculi running in the centres of the columns of liver cells. ×230.

FIG. 5.—The same field taken a few sec. later. Here, the objective has been focused more on the continuous sheet of liver cells just beneath the capsule. The canaliculi of these cells form a network with branches which communicate with the intracolumnar canaliculi beneath them, some of which can be seen. ×230.

FIG. 6.—Another field of the same liver half an hour later. The liver cells contain less fluorescein and the canaliculi are even more brilliant. ×230.

FIG. 7.—The liver in severe hypoxia. The animal had been given 7 per cent oxygen in nitrogen for an hour and the picture was taken 10 min. after fluorescein had been injected. The liver cells around the "central" vein contain little fluorescein and are not secreting. Peripheral cells are secreting, and in the cells of the boundary zone vacuoles filled with fluorescein are present. ×230.

FIG. 8.—Paraffin section of liver of a rat killed 1 hr. after being subjected to 7 per cent anoxia for 2 hr. The liver had been viewed under the fluorescence microscope. In the lobules beneath the capsule, the nuclei at the centres are pyknotic and the cytoplasm pale staining. This is a centrilobular necrosis. H and E, ×130.

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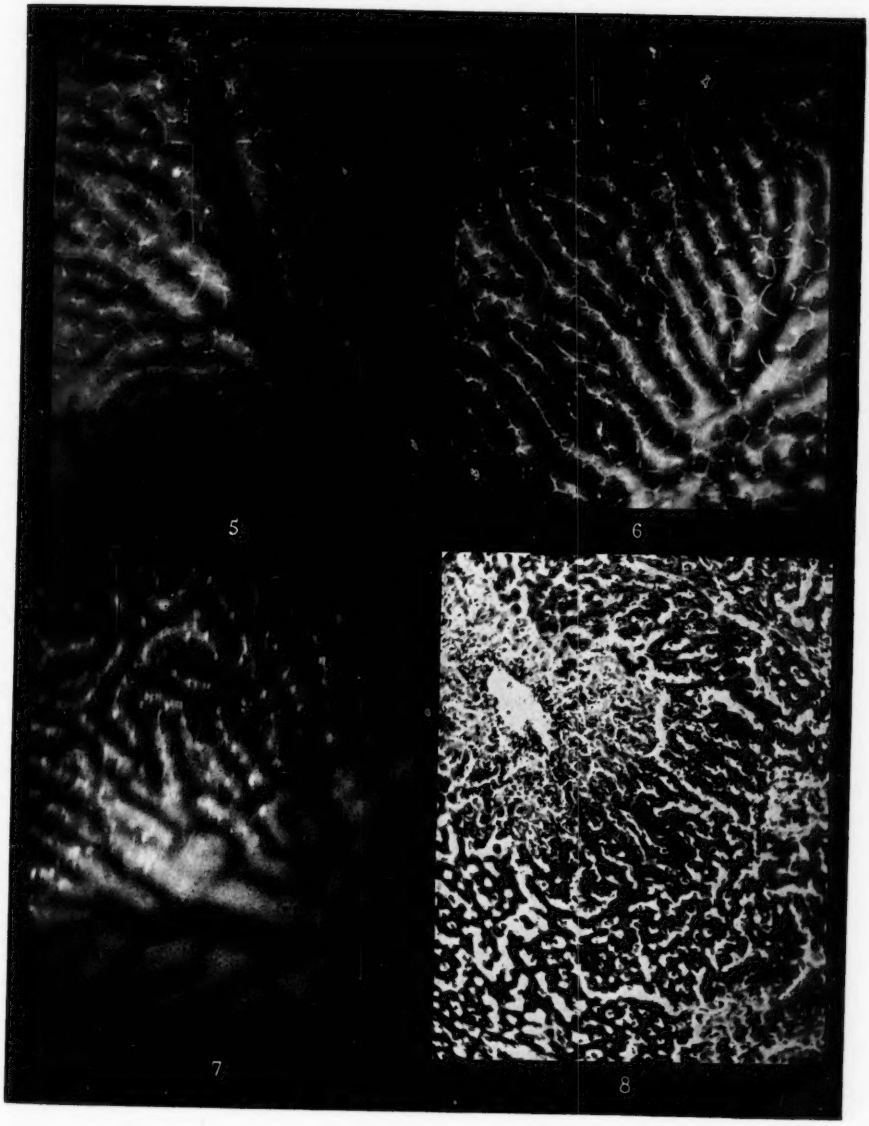

Reese.

Because the secretion of fluorescein is a vital phenomenon and not a mere passive transference, temperature control is of great importance. Hanzon found that secretion was slowed by low temperatures and accelerated by high temperatures. Between the range 25–44°, the speed followed the formula which Arrhenius (1889) found applied to chemical reactions. At 38°, Hanzon found that the average time taken for the dye to pass from the sinusoid to the liver cell was 22·5 sec. (SD = 5·3) and from the sinusoid to the bile canaliculus was 46·5 sec. (SD = 11·5). His first figure is twice that of the corresponding figure in Table I of this work, but as the end-point for liver-cell fluorescence is far from sharp, the difference is probably subjective. The first appearance of bile canaliculi is much more easily timed, and the fact that our second figures are similar would indicate that the method is capable of repetition under standard conditions in different laboratories.

With severe hypoxia, produced either by tying the hepatic artery or creating a state of haemorrhagic shock, a little fluorescein reaches the liver cells but concentration in the bile canaliculi is completely suppressed. With moderate hypoxia, fluorescein is taken up by the liver cells and secreted into the canaliculi but more slowly than usual. The time taken for the dye to appear in the canaliculi after reaching the sinusoids is about twice normal. This state of affairs is reversible, and when the hypoxia is relieved, a second dose of dye is secreted in the normal time. Others have noted the slowing of biliary flow with hypoxia. Tanturi and Ivy (1938) gave mixtures of 10 and 7·5 per cent oxygen in nitrogen for 20 min. at a time to 4 dogs. In 3 of them the biliary flow was normal, but in the 4th it was reduced by a half after 6 min. of hypoxia. Schnedorf and Orr (1941) gave mixtures of 20, 15, 10 and 5 per cent oxygen in nitrogen for 30 min. to anaesthetized dogs and found a 16–50 per cent decrease in biliary secretion with 15 per cent oxygen. This decrease was more marked with 10 and 5 per cent oxygen, and acholia developed in 2 animals. Chardon, Neverre and Jeannoel (1949) made dogs inhale 3 per cent oxygen in nitrogen. The bile flow fell from 7 drops to 3 drops per min. after 3 min. of this drastic treatment but it was restored to normal when the dogs were allowed to breathe air. Engstrand (1949) noted a decrease in the secretion of bromsulphthalein in the bile of cats and dogs breathing 9 per cent oxygen in nitrogen for 20 min., and this was accompanied by a decrease in the volume of bile secreted.

When rats breathe 10 or 11 per cent oxygen, the whole liver often shows no abnormality other than the slowing of the secretion. When less oxygen than this is given, although the peripheral cells of the lobule secrete biliary fluorescein, the more centrally placed and therefore more hypoxic cells cannot do so. Between the zones of proper secretion and the zones where secretion is suppressed, the cells develop vacuoles in the cytoplasm which fill up with dye. Vacuoles containing neither fat nor glycogen were noted in the liver in necropsies on members of air crew who died during the Second World War as the result of high altitude flying. Mueller and Rotter (1942) in Germany, and Kritzler (1944) and Lewis and Haymaker (1948) in the United States reported such necropsies in their respective Air Forces. Hesse (1942), Kritzler (1944) and Gillman and Gillman (1948) found similar vacuoles in cases who died of hypoxia from other causes, *viz.*: carbon monoxide and prussic acid poisoning, drowning and suffocation, but they were not found in cases of sudden death. These observations stimulated pathologists to subject laboratory animals to hypoxia either by reducing the atmospheric pressure or diluting air with nitrogen. Pichotka (1942), Florentin, Grandpierre,

Grognot and Royer (1944) and Trowell (1943) all produced liver-cell vacuoles by these methods. Trowell (1946) made further studies on the formation of these vacuoles. He found that they developed *in vivo* in animals made hypoxic for 20–30 min. and *post mortem* in the first 5–15 min. provided that the animal was not bled out. Vacuoles were clearly visible in fresh liver cells mounted in the animal's own heparinized plasma, and if the cells were ruptured, the granular cytoplasm might break away in fragments leaving the vacuoles as discrete bodies floating in the plasma. He wrote " ... when oxygen supply to the liver is seriously impaired, water passes from the blood into the liver cells and forms large watery vacuoles in the cell cytoplasm. This phenomenon only occurs provided that, during the period of liver anoxia the blood pressure in the liver sinusoids is maintained at least at its normal value ; if the pressure is above normal, the liver cell vacuolation is more rapid and greater in extent. It seems that anoxia, by increasing the permeability of either the sinusoids or the liver-cell walls, allows the existing hydrostatic pressure in the sinusoids to force water and probably other plasma constitutents into the liver cells ... ". Anyone who has observed these vacuoles develop under the fluorescence microscope will be unable to accept Trowell's explanation but will agree with Hanzon that water and biliary constituents penetrate the cells from the bile capillaries.

When the rat is rendered severely hypoxic, an irreversible change takes place in the cells at the centres of the lobules. Autofluorescence in these cells is diminished and they no longer secrete fluorescein. Even after the relief of the hypoxia, the cells around the central vein no longer function. Histological sections show centrilobular necrosis in lobules beneath the capsule (Fig. 8), and it seems likely that this is caused by the small dose of ultra-violet light acting on severely hypoxic cells. Bywaters (1946) reported necrosis in a high proportion of livers from cases of crushing injury and skeletal trauma from other accidents. The necrosis appeared histologically to date from the time of injury, and there were hydropic non-fatty vacuoles in some of the liver cells similar to those described by Kritzler and others in hypoxic conditions. If hypoxia alone does not cause hepatic necrosis, perhaps relatively hypoxic liver cells are more liable to necrosis than those with a better oxygen supply. This may be why centrilobular necrosis appears in many toxic and infective conditions.

SUMMARY

The secretory function of the liver was studied in the rat by the method of intravital fluorescence microscopy. The surface of the liver was observed under the microscope with violet or ultra-violet incident light illumination. A solution of sodium fluorescein was injected intravenously and appeared in the hepatic sinusoids. It was soon taken up by the liver cells and was concentrated in the bile canaliculi which became brilliantly fluorescent. This active secretory process resembled the normal elimination of bilirubin from the plasma to the bile.

Hypoxia, produced by reducing the oxygen content of the inspired air to 10 or 11 per cent, merely slowed down the rate of secretion. More severe hypoxia caused the centrilobular cells to stop secreting altogether. Surrounding these inactive centrilobular cells, there appeared cells with abnormal vacuoles in the cytoplasm similar to vacuoles noticed by others in the livers of humans and animals which died of hypoxia. These vacuoles were strongly fluorescent and contained biliary fluid. With severe hypoxia, some of the centrilobular cells

underwent necrosis. The relative hypoxia of centrilobular cells might be the reason for centrilobular necrosis in many toxic and infective conditions.

My thanks are due to Professor G. J. Cunningham, M.B.E., in whose department this work was carried out. Professor D. Slome and Professor R. F. Woolmer, V.R.D., kindly lent me essential apparatus. Dr. J. F. Nunn encouraged me throughout and advised me on all matters concerning respiration. Dr. V. Hanzon of Uppsala gave me the benefit of his great experience of the method. Dr. E. Marley taught me to cannulate the vessels of the rat. Mr. R. D. Chambers and Mr. A. L. E. Barron helped me with the photography.

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NEPHROCALCINOSIS IN THE RAT: EFFECT OF INJECTING CHONDROITIN SULPHATE

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THE nephrocalcinosis produced in the rat by parathyroid extract cannot be explained solely by an increase of calcium in the blood and urine (Grimes, 1957; Fourman, 1959). The calcium deposits are seen to be associated with a material having the histochemical characteristics of a polysaccharide. Engel and Catchpole (1953) suggested that this material was derived from mucoproteins released from bone matrix by parathyroid hormone. Shetlar, Howard, Joel, Bowling and Shetlar (1958) found that in rats parathyroid extract increased the serum levels of glycoproteins and seromucoid before there were any lesions in the kidney. Baker and Sison (1954) described thickened basement membranes and lesions containing polysaccharide material without calcium deposits in kidneys from rats given small doses of parathyroid extract for 2 weeks. They concluded that alterations of ground substance preceded and were necessary for calcium deposition. It is not clear whether the renal changes result solely from the passage through the kidney of material released from bone matrix or whether parathyroid hormone also acts directly on the connective tissue of the kidney. The present study was undertaken to see if the changes could be reproduced by injecting a mucopolysaccharide, and if these injections rendered the kidney more susceptible to calcification. Chondroitin sulphate (CS) was the material injected as it has been suggested that this acid mucopolysaccharide plays an important role in calcification (Rubin and Howard, 1950; Sobel, 1955). Three chondroitin sulphates are distinguished on the bases of solubility, optical rotation and enzymatic hydrolysis (Meyer, Hoffman and Linker, 1957). Two of these, designated A and C, are found both in bone and cartilage, but cartilage CS which was used in these experiments contains a smaller proportion of chondroitin sulphate C than does bone.

When injected subcutaneously into rats CS has been shown to be excreted in the urine (Dohlman, 1956). Tinacci and Benassi (1954) gave rats single intravenous injections of CS and found an increase of metachromasia in the kidney at the level of the papilla.

MATERIAL AND METHODS

The animals were male rats of the MRC hooded strain, aged 14–18 weeks and weighing 137–300 g. They were fed on "Oxoid rat cubes" and given water *ad libitum*. Each pair of experimental and control animals was drawn from the same litter.

Seven rats were injected subcutaneously twice daily with parathyroid extract (Eli Lilly).

Twelve rats were given daily subcutaneous injections of a solution of physiological saline containing 10 mg. potassium chondroitin sulphate (K.CS) per ml. The K.CS was prepared

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by the method described by Dodgson, Lloyd and Spencer (1957). Its purity was confirmed by Dr. A. G. Lloyd ; N/S ratio was 1·08.

Six rats were given daily intraperitoneal injections of 1·5 ml. of a 10 per cent solution of calcium gluconate. Each injection contained 13 mg. calcium.

Six rats were given injections of both K.CS and calcium gluconate.

The control animals were injected with the same volume of physiological saline.

Rats were killed by exsanguination under light ether anaesthesia and in some serum was separated for the estimation of calcium.

One kidney was used for chemical analysis of calcium. Portions of the other kidney were fixed in formalin buffered to pH 7·6 with veronal, in cetyl pyridinium chloride 0·5 per cent in 4 per cent aqueous formaldehyde (Williams and Jackson, 1955), and in cold 80 per cent alcohol, and then embedded in paraffin.

Sections (6 μ) from experimental and control animals were mounted on the same slide. Metachromasia was demonstrated by Azure A (Kramer and Windrum, 1955) and toluidine blue (Pearse, 1953). Acid mucopolysaccharides were selectively stained with Alcian blue using neutral red as a counterstain (Pearse, 1953). Neutral polysaccharides were stained by the periodic-acid-Schiff (PAS) method of McManus (1948). Calcium was demonstrated by the von Kossa method modified so that the PAS method could be used on the same section (Moffat, 1958). Other sections were stained with haematoxylin and eosin, Gomori's modification of Wilder's method for reticulin and Gomori's method for alkaline phosphatase.

Calcium in the serum and kidneys was estimated by the method of Sobel and Sobel (1939). The calcium contents of kidneys are expressed as mg./g. of fat free dry kidney.

RESULTS

Controls

The calcium content of the kidneys ranged from 0·2–0·5 mg./g. No calcium or increase of metachromatic or Alcian blue staining material was demonstrated in sections. There were no intratubular casts.

Experiment A

One rat was injected with 50 units of parathyroid extract twice daily (total 800 units). The calcium content of the kidney was 16·9 mg./g. Calcium was deposited in all parts of the kidney, but most of it was in the cortex, and the inner zone of the cortex was more heavily calcified than the outer. In the outer cortex there were calcium deposits in the basement membranes and in cells of the proximal convoluted tubules. In addition there were intratubular casts of calcium, large interstitial deposits of calcium in the cortex and smaller casts and interstitial deposits in the medulla (Fig. 1–3). The distal convoluted tubules and many of the proximal convoluted tubules were dilated. Their basement membranes were thickened and stained deeply with PAS and the brush borders of the proximal convoluted tubules were broken up and showed loss of alkaline phosphatase activity. Most of the thickened basement membranes were not calcified. There were in the cortex areas of increased interstitial tissue containing fine PAS-staining reticular fibrils and staining deeply with Alcian blue. Many such areas stained metachromatically and contained calcium. All casts stained with PAS, some uncalcified casts had phosphatase activity ; but only calcified casts stained with Alcian blue and showed metachromasia. Intensity of Alcian blue staining and metachromasia was increased in the glomeruli and in the interstitial tissue of the lower medulla (Fig. 1).

Experiment B

Six rats were given twice daily injections of parathyroid extract in doses of 10,

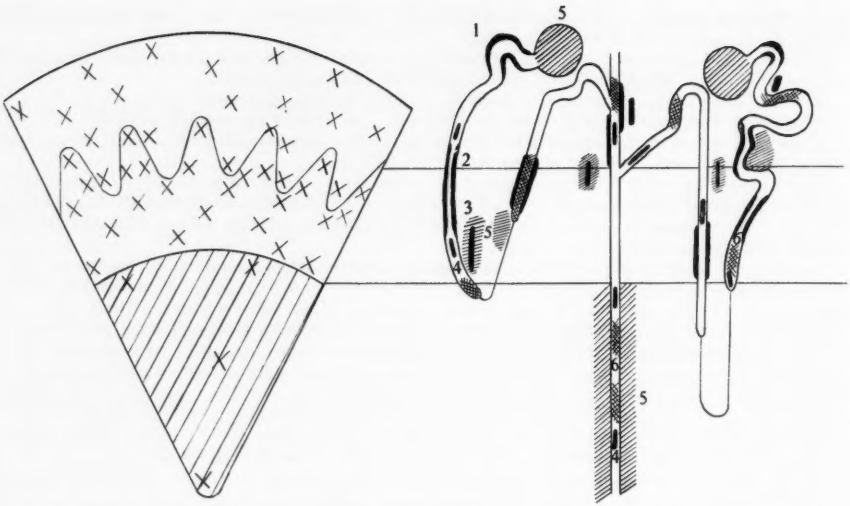


FIG. 1.—The distribution of calcium deposits and changes of mucopolysaccharide staining in the kidney of a rat given 800 units of parathyroid extract. Calcium in tubular basement membrane (1) and cells (2), in the interstitial tissue (3) and as casts (4) is represented by heavy black lines. Crosses indicate sites of calcium deposits seen in section. An increase of Alcian blue staining in glomeruli and interstitial tissues is represented by hatching (5) and PAS positive casts by cross hatching (6).

20 or 50 units. The total amount of extract given ranged from 150–340 units. The calcium content of the kidneys ranged from 0·4 to 1·8 mg./g. Two were within the normal range. Serum calcium was raised by 1–2 mg.

In kidneys from only 2 animals was calcium demonstrated histochemically. These rats had both received in 20 unit doses a total of 200 units of hormone. In 4 kidneys there were small eosinophilic, PAS-positive casts which were not metachromatic and did not stain with Alcian blue (Fig. 5). In 5, the normal Alcian blue staining and metachromasia of the glomeruli and the interstitial tissue of the lower medulla were intensified (Fig. 4). The sections were otherwise normal compared with controls. No alteration was observed in the basement membranes, and alkaline phosphatase activity was not reduced.

Experiment C

Twelve rats were injected daily with K.CS in doses of 5–20 mg. for 8–23 days. The total amount of K.CS given ranged from 40 to 220 mg. The calcium content of the kidneys was within the normal range, and no calcium was demonstrated histochemically in the kidneys of these animals. In the collecting ducts of 8 kidneys there were small eosinophilic, PAS-positive casts which were not metachromatic and did not stain with Alcian blue (Fig. 6). In kidneys from 10 of these rats the normal Alcian blue and metachromatic staining of the glomeruli and of the interstitial tissue in the lower medulla was intensified (Fig. 4). These changes were not related to the dose of K.CS or to the period over which it was given.

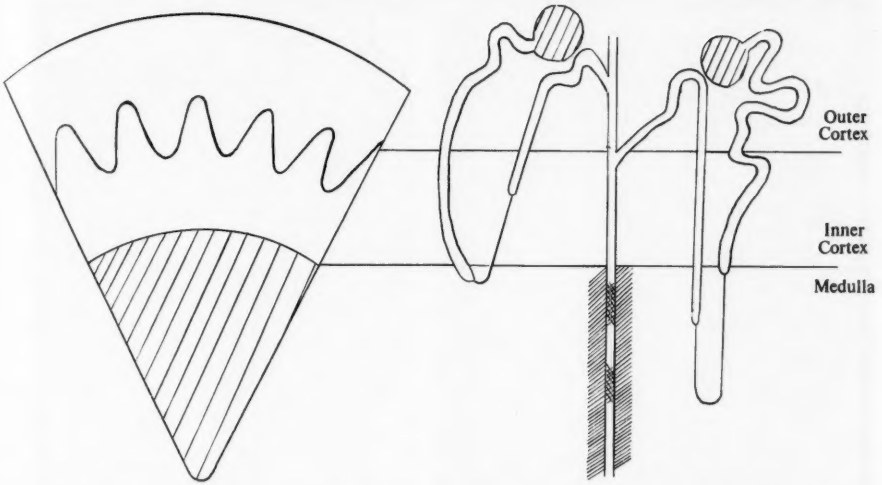


FIG. 4.—Changes of mucopolysaccharide staining in kidneys of rats given either K.CS or small doses of parathyroid extract. Increase of Alcian blue staining in glomeruli and interstitial tissue of the medulla is shown by hatching. Cross hatching in the collecting ducts indicate PAS-positive casts.

Experiment D

Six rats were injected daily with 13 mg. Ca as the gluconate, for 8–23 days. The calcium content of the kidneys of these animals ranged from 3·3 to 12·1 mg./g. (mean 6·8 mg./g.). The serum calcium was raised by 1–2 mg. The urinary calcium has previously been shown to be raised by calcium gluconate injections (Fourman, 1959). Calcium deposits were demonstrated histologically in sections of all these kidneys. They were present only in the outer zone of the cortex and confined to the basement membrane of the first part of the proximal convoluted tubules as previously described (Fourman, 1959). In the kidney containing 12·1 mg./g. of calcium, heavily calcified basement membranes were thickened. These stained brilliantly with Alcian blue and deeply with PAS but they were not metachromatic. Uncalcified basement membranes were not thickened and there were no intratubular casts and no increase of staining with Alcian blue or of metachromasia in the glomeruli or medulla.

Experiment E

Six rats were given two injections daily for 8–23 days, one of 13 mg. Ca as the gluconate and the other of K.CS in doses of 5–20 mg. The total amount of K.CS given ranged from 110 to 180 mg. The calcium content of the kidneys ranged from 3·2 to 9·4 mg. (mean 7·1 mg./g.). The serum calcium was raised by 1–2 mg. Calcium deposits were demonstrated in sections of all these kidneys. The calcium was present solely in the outer zone of the cortex in the basement membrane of the first part of the proximal convoluted tubule (Fig. 7 and 8). Basement membranes were not thickened. There were in the collecting ducts of 3 of the kidneys

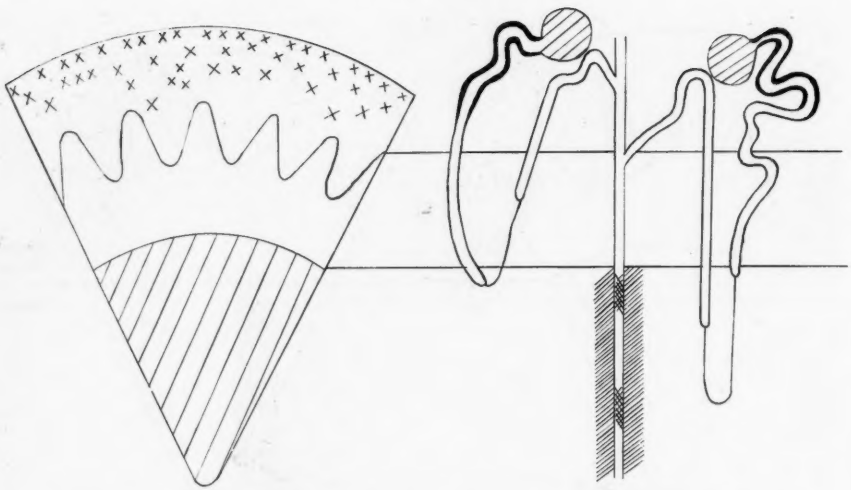


FIG. 8.—The distribution of calcium deposits and changes in mucopolysaccharide staining of the kidney in rats injected with calcium gluconate in addition to K.CS. Calcium is in the outer cortex, solely in the basement membrane of the first part of proximal convoluted tubules as in rats given calcium gluconate alone. Crosses indicate sites of calcium deposits in sections. Heavy black lines indicate calcified basement membrances. Increase of Alcian blue staining in glomeruli and interstitial tissue of the medulla is shown by hatching. Cross hatching in the collecting ducts indicates PAS-positive casts which contain no calcium.

casts which did not stain with Alcian blue and were not metachromatic (Fig. 8). These casts were PAS positive. They were not calcified. In 5 of these kidneys there was an increase of Alcian blue staining and of metachromasia in the glomeruli and lower medulla.

DISCUSSION

Large doses of parathyroid extract, such as were given to our first animal, produce two types of casts in the renal tubules. The first is metachromatic and contains calcium ; the second is not metachromatic and does not contain calcium. When small doses of parathyroid extract were given only the second type of cast was produced. Baker and Sison (1954) suggested that these hyaline casts are precalcification lesions and Engfeldt, Gardell, Hellström, Ivemark, Rhodin and

EXPLANATION OF PLATE

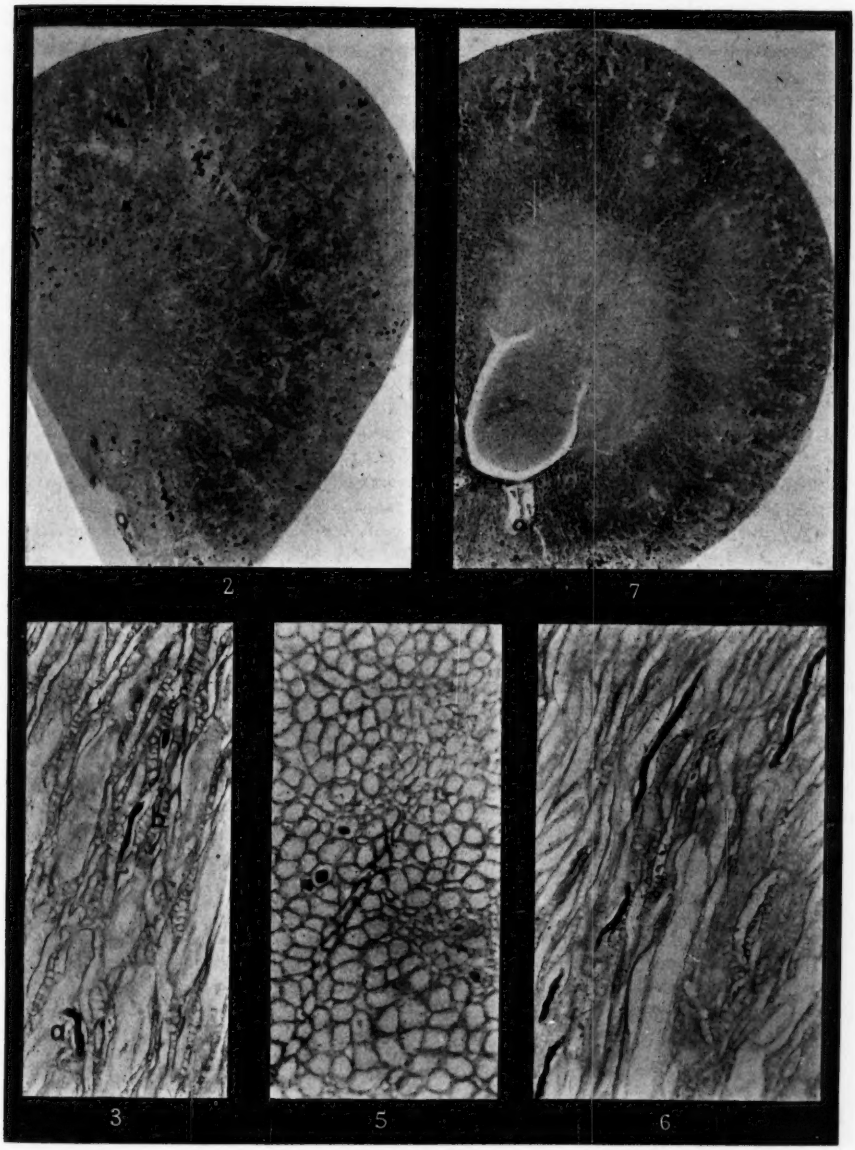
FIG. 2.—Section of kidney from a rat given 800 units of parathyroid extract. Black deposits of silver at site of calcium deposits. von Kossa. PAS. ×7.

FIG. 3.—Section of renal medulla from a rat given 800 units of parathyroid extract. The lowest cast (*a*) contains calcium and has a black granular appearance. The three other casts (*b*) are uncalcified PAS-positive (red) and homogeneous. von Kossa. PAS. ×130.

FIG. 5.—Section from renal medulla of a rat given 200 units of parathyroid extract. In the collecting ducts are PAS-positive casts containing no calcium. von Kossa. PAS. ×130.

FIG. 6.—Section from the renal medulla of a rat given K.CS. In the collecting ducts are PAS-positive casts containing no calcium. von Kossa. PAS. ×130.

FIG. 7.—Section from the kidney of a rat given injections of both K.CS and calcium gluconate. Fine black deposits of silver at the site of calcium deposits are seen solely in the outer zone of the cortex. von Kossa. PAS. ×7.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Strandh (1958) have shown that PAS positive casts may serve as a mucoprotein matrix for the precipitation of crystals. Shetlar *et al.* (1958) found that PAS-positive uncalcified casts appear later than the calcified ones, and considered that they might consist of serum protein products found in the tubules after injury to the kidney. When we gave small doses of parathyroid extract, hyaline casts appeared without calcium deposits and the kidneys showed no evidence of damage; the brush borders of the proximal convoluted tubules appeared normal and the casts showed no alkaline phosphatase activity. In kidney sections from the rat which was given 800 units of parathyroid extract, the casts containing alkaline phosphatase may have been formed from disintegrated brush borders. Addis (1928) suggested that renal hyaline casts might be urinary protein precipitated in combination with CS. Such might well be the origin of the casts we have described, as these were seen after CS injections. The association of protein with the CS would explain why the casts were not metachromatic, for metachromasia of mucopolysaccharides may be inhibited by protein (French and Benditt, 1953; Hammerman and Schubert, 1953). Spicer and Bryant (1958) gave rabbits large intravenous injections of CS and observed metachromatic granular casts in the collecting tubules of the kidney. They used acidic fixatives which may have unmasked the metachromasia by dissociating the polysaccharide protein complex.

We did not see thickening or alteration of polysaccharide staining of the kidney basement membranes after small doses of parathyroid extract, although in von Kossa preparations of 2 out of 4 kidneys with raised calcium content there were occasional fine black lines in some basement membranes. These results do not support the postulate of Baker and Sison (1954) that an alteration of the basement membrane is necessary for subsequent calcification. Small doses of parathyroid extract did however produce casts and those changes in glomeruli and medullary connective tissue which are associated with parathyroid nephrocalcinosis. These changes could be reproduced by injections of CS; they may therefore be the result of the passage through the kidney of CS released from bone. The injections of CS did not however render the kidney more susceptible to calcification even in the presence of increased levels of calcium in blood and urine. These results do not decide what role, if any, an increase of mucopolysaccharide excretion plays in the production of nephrocalcinosis by parathyroid extract.

SUMMARY

CS injected into rats produces casts in the collecting tubules and an increase of Alcian blue staining material in the glomeruli and in the interstitial tissue of the medulla. These changes are also produced by injection of small doses of parathyroid extract; CS injections do not however affect the calcium content of the kidney nor do they modify the changes induced by the injection of calcium gluconate.

I should like to express my thanks to Professor J. S. Baxter and Professor H. Scarborough for their interest and encouragement and to Dr. P. Fourman for his advice and help in this work. It was carried out with the aid of a grant to Dr. P. Fourman from the Empire Rheumatism Council. I am most grateful to Dr. F. Jacoby for his helpful suggestions and criticisms of this manuscript and to Dr. A. Lloyd for his help in the preparation of the chondroitin sulphate used. I am

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THE THROMBOGENIC PROPERTIES OF HUMAN SERUM: A BIOASSAY

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WESSLER (1955) produced intravascular coagulation in the occluded veins of dogs following the systemic infusion of serum fractions, rich in convertin (Factor VII), one of the factors accelerating the conversion of prothrombin to thrombin (Alexander, de Vries and Goldstein, 1948).

Such serum fractions give rise to a transient hypercoagulability of the blood of the recipient animal (Wessler, 1955) and, in increasing doses, progressively shorten the coagulation time down to a basal level (Scott and Solis, 1959).

The exact cause of this hypercoagulability is still obscure. Originally it was thought that convertin alone might be responsible (Wessler, 1955) but this has not been confirmed by subsequent investigation, which has suggested that other factors, possibly acting in conjunction with convertin and as yet unidentified, are involved (Wessler, 1957).

Scott (1959) demonstrated a difference in the thrombogenic properties of two convertin-rich serum fractions by comparing the amount of soft red thrombus produced in the occluded portal veins of successive rabbits receiving progressively increasing doses of one or other fractions.

This suggested a method for investigating the thrombogenic properties of these serum fractions as well as whole serum quantitatively, *in vivo* rather than *in vitro*, and a broad preliminary study has been carried out along these lines.

MATERIALS AND METHODS

Batches of pooled human serum, varying in volume from 200–400 ml., and in age from 1–5 days, were used. These were for the most part divided into several portions, convertin-rich fractions being produced from some, some being frozen for subsequent use, while others were freeze-dried and later reconstituted in varying amounts of distilled water.

Convertin-rich fractions were manufactured by the method described by Wessler (1955). After adsorbing the serum with barium sulphate (100 mg./ml. of serum) and discarding the supernatant fluid, the barium sulphate was washed with an acetate buffer (pH $\pm$ 5·5) and eluted twice with a volume of 5 per cent sodium citrate equal to one-eighth of the original volume of serum. The resultant fraction, henceforth referred to as eluate, was dialysed overnight against either tap or distilled water and frozen until required. All frozen material was thawed at room temperature immediately before use.

Scott (1959), investigating the haemodynamics of sudden portal vein occlusion in the rabbit, deduced that a variable, and sometimes considerable movement of blood occurred in the vein after occlusion, due partly to the vigorous intestinal peristalsis. In the rat, intestinal peristalsis can seldom be observed with the naked eye, suggesting that the movement of blood within the portal vein after occlusion is likely to be less than in the rabbit, and for this reason the rat was chosen.

Male Wistar rats, weighing 150–300 g. were anaesthetized and after opening the peritoneal cavity, the mesentery was spread out and the portal vein exposed.

A simple formula was devised in order to produce graduated concentrations, over a wide

range, of either eluate or serum in the circulating blood of the rats. The blood volumes of the rats were estimated on the basis of 4·3 ml./100 g. of body weight (Rowett, 1957) and both serum fractions and sera were assumed to contain 200 units of "coagulability" (C units) per ml., this particular figure being chosen as it produced easily measurable doses for injection.

Thus, if x = weight of the rat in grammes, y = volume of material injected in ml. and z = number of C units to be produced per ml. of circulating blood of the recipient animal, then :

$$z = \frac{200y}{0{\cdot}043x + y}$$

$$200y = 0{\cdot}043xz + yz$$

$$200y - yz = 0{\cdot}043xz$$

$$y = \frac{0{\cdot}043xz}{200 - z}$$

The required volume of either eluate or serum was injected into the inferior vena cava and a light clamp placed immediately on the portal vein close to the liver. After 10 min. occlusion, with the clamp still in place, the portal vein was decompressed by opening one or more of its small tributaries so as not to dislodge small fragments of thrombus. The main vein was then opened and the amount of thrombus formed was assessed.

Thrombus formation varied from four degrees, where thrombus filled the whole length of the vessel, to one degree where a quarter of the latter was occupied, lesser amounts being recorded as a half degree.

Electrophoresis of eluates and sera was performed on 3 MM Whatman paper for 18 hr., using barbitone buffer (pH 8·6), ionic strength 0·083. The strips, to which 0·1 ml. of the material concerned had been applied, were stained with naphthalene black, 12B.

RESULTS

All eluates, whether dialysed against tap or distilled water, behaved in a similar manner. No thrombus was produced below a certain concentration of C units, while once thrombus had been formed, it increased rapidly in amount until it became either massive or total in successive animals (Fig. 1).

The lowest concentration of C units which produced thrombus was remarkably

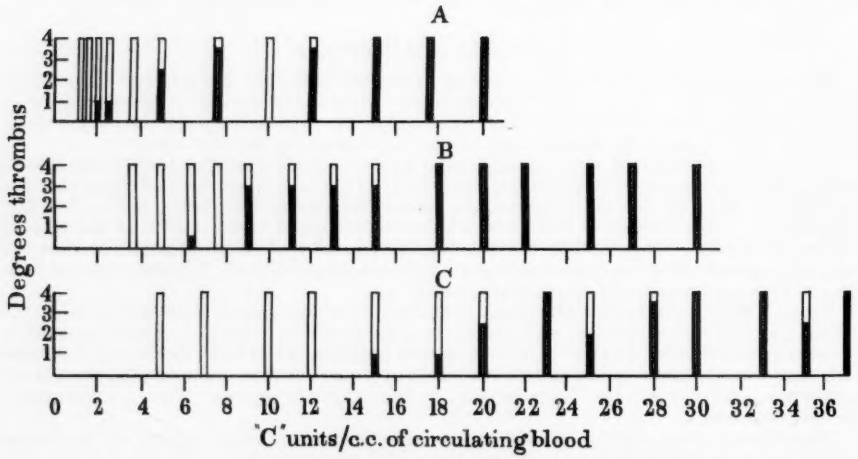

FIG. 1.—Thrombus production by 3 serum eluates in the portal veins of rats.

constant for any one eluate, thrombus being formed a minimum of two out of three times at the same concentration.

Two eluates, prepared from different sera, were divided into equal volumes, which were dialysed against tap and distilled water respectively and the volumes of each pair of samples equalized after dialysis by the further addition of distilled water. The lowest concentration of C units producing thrombus was virtually the same with both types of eluates, the dialysing medium having no demonstrable effect on their potency.

The volume by which eluates increased during dialysis was varied by adjusting the slack in the dialysis bag before dialysis began. By the further addition of distilled water where necessary, a series of eluates was produced whose volumes had been increased, during and after dialysis by 40–90 per cent.

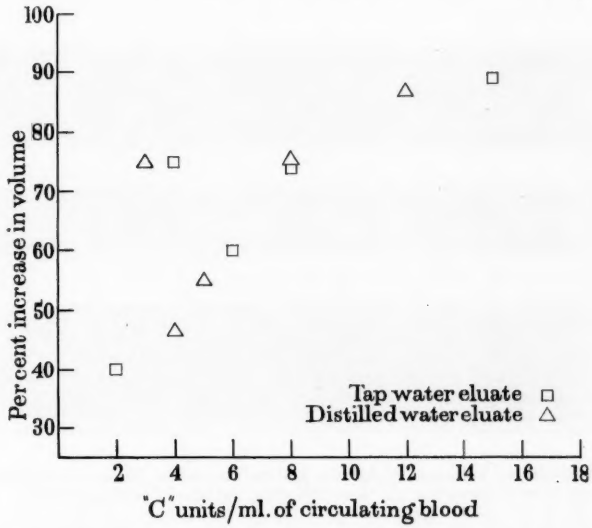

FIG. 2.—The relationship of the dilution of human serum eluates to their potency.

The lowest concentration of C units capable of producing thrombus was determined for each eluate and, with one exception, this bore a broad inverse relationship to the percentage increase in volume the eluate had undergone, eluates prepared from different sera as well as those prepared from the same serum and diluted by differing amounts behaving in this manner (Fig. 2).

Electrophoresis of human serum before and after adsorption with barium sulphate, did not reveal any alteration in the protein composition, but the resultant eluates, concentrated 30 times, were found to contain albumin and globulin in varying proportions (Fig. 3). All eluates contained α_2 globulin, while β globulin was present in most and one contained a trace of the α_1 fraction. The proportions of these fractions varied and no constant electrophoretic pattern was obtained, while the total amount of protein present also varied and was not related to the dilution of the eluate.

The thrombogenic properties of an eluate, compared with those of the original serum (Fig. 4) showed that the latter was not only infinitely less potent in producing thrombus but the subsequent rapid and progressive increase in thrombus formation, produced by the former in increasing doses, did not occur.

A batch of serum was divided into 3 parts, an eluate being prepared from one, another being freeze-dried and reconstituted in a third of its original volume of distilled water, while the third was frozen and later thawed. The concentrated serum initiated thrombus formation at approximately one-third of the concentration of C units as did the original serum (Fig. 5). Although the former first produced thrombus at a lower level of C units than the eluate, the subsequent constant production of massive or total thrombosis seen in successive rats receiving eluate was not observed in animals in which similar concentrations of C units were produced by the concentrated serum (Fig. 6).

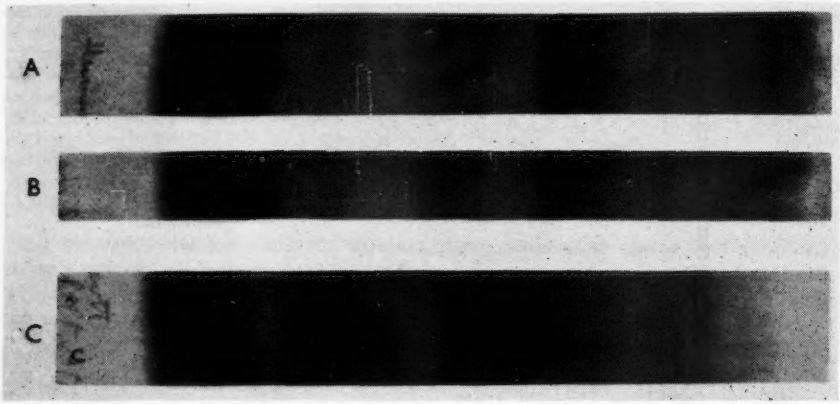

FIG. 3.—Electrophoretic patterns of human serum (*a*) before barium sulphate adsorption, (*b*) after adsorption; (*c*) electrophoretic pattern of resultant eluate.

Freeze-dried serum reconsituted to its original volume with distilled water was of almost similar potency to the original serum, which had been frozen only (Fig. 7).

The amounts of thrombus produced by various sera in successive animals showed a peculiar periodicity (Fig. 5 and 6) and where batches of the same serum were compared, the maximum thrombus production occurred at almost identical concentrations (Fig. 7).

DISCUSSION

Human serum eluates and pooled sera, injected into rats in sufficient quantities, are thrombogenic, producing intravascular coagulation in the occluded portal vein.

An arbitrary unit of coagulability, the C unit, was devised and assuming that all the materials injected contained the same number of C units per ml., increasing concentrations of this unit were produced in the circulating blood of the recipient animals depending on their weights and blood volumes.

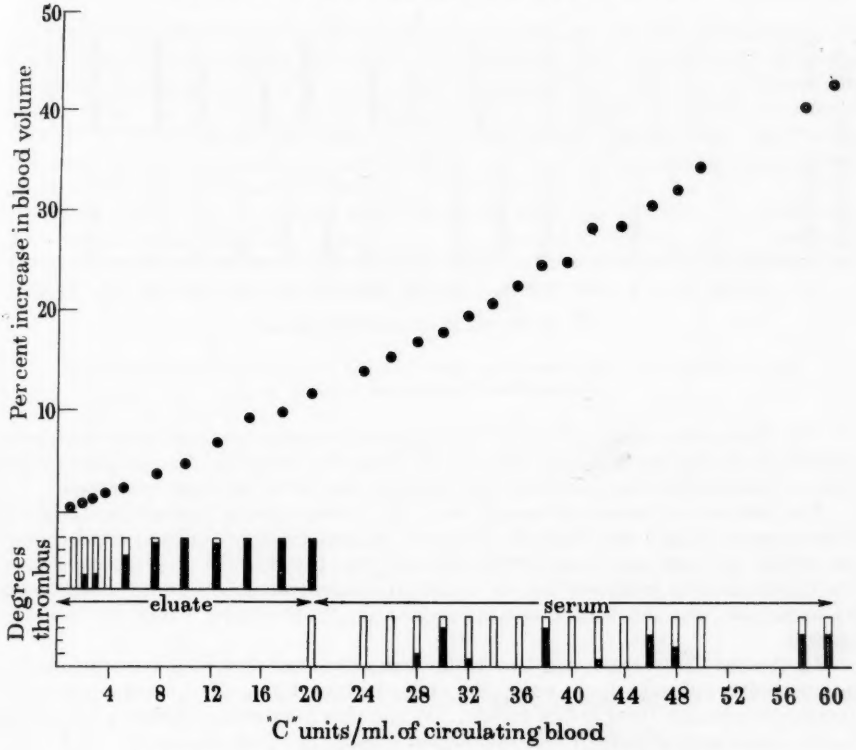

FIG. 4.—Thrombus formation by an eluate and its original serum.

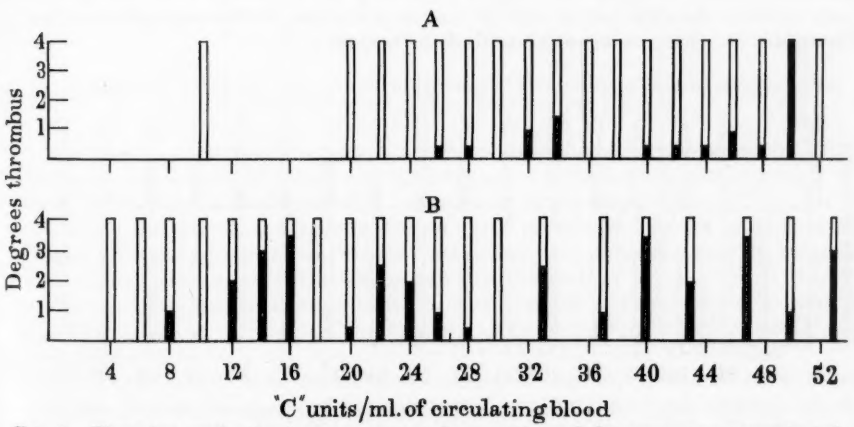

FIG. 5.—Thrombus production by unconcentrated serum (A), and the same serum concentrated to one-third of its original volume (B).

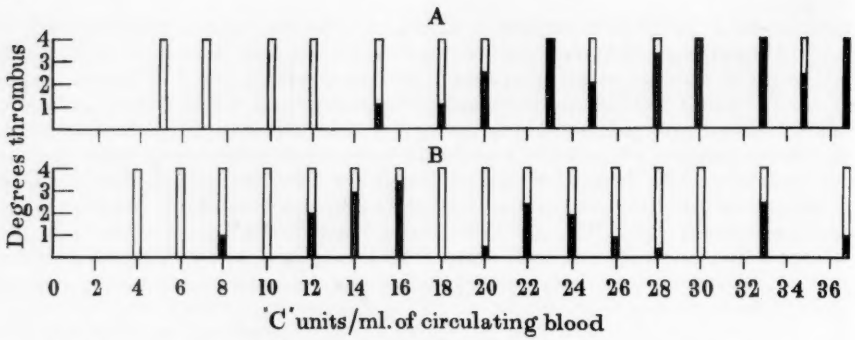

FIG. 6.—Thrombus production by an eluate (A), and its original serum concentrated to one-third of its original volume (B).

The thrombogenic properties of various serum eluates and sera were compared quantitatively by assessing the amount of thrombus formed in the occluded portal veins of successive rats following the systemic injection of these materials.

The pattern of thrombus formation in the portal vein of the rat produced by human serum eluate was basically the same as that reported in the portal vein of the rabbit by Scott and Solis (1959) following the injection of dog serum eluate. No thrombus was produced up to a certain concentration of eluate after which its formation was inconstant and irregular, finally becoming complete in every animal.

In the rat, however, once thrombus formation had commenced, its increase in successive animals up to complete and constant filling of the vein was more evenly progressive than in the rabbit. While this may reflect either a difference in the behaviour of human and dog serum eluates or of the blood of rabbits and rats, it may be confirmation of the probable difference, already mentioned, in the haemodynamics of the portal venous fields of these 2 animals after occlusion and may indicate that the portal vein of the rat is a more reliable vehicle than that of the rabbit in which to assess thrombus formation.

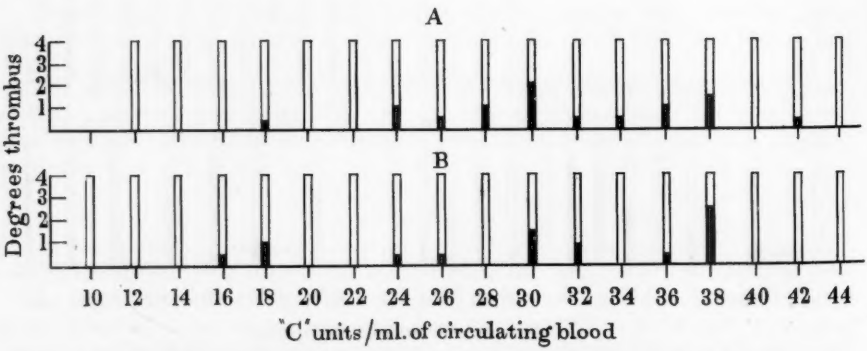

FIG. 7.—Thrombus production by frozen serum (A), and freeze-dried serum (B).

The lowest concentration of C units required to produce thrombus using any one eluate was remarkably constant. The same serum first produced thrombus at almost the same level of C units after freezing or freeze-drying, while a serum concentrated to one-third of its original volume was approximately 3 times as potent as in its unconcentrated form. These facts indicate that the results obtained by this procedure are reproducible, and that differences between various eluates and sera were not due to experimental variation.

There was a broad inverse relationship between the dilution of eluates and their potency (Fig. 2), the one exception encountered being due more probably to the undue potency of the original serum than to its freshness, other eluates prepared from equally fresh sera behaving in the usual manner on dilution.

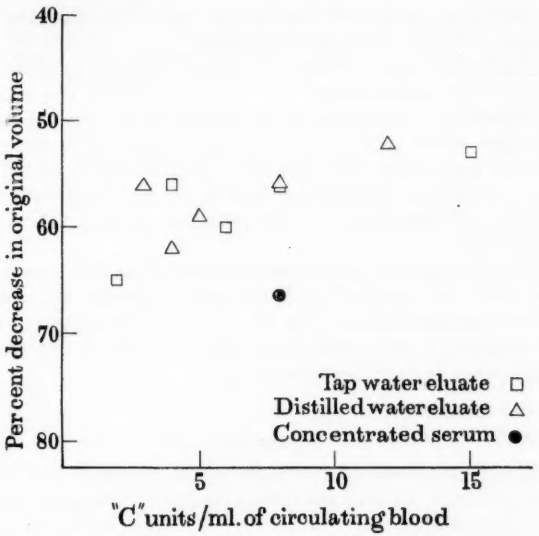

FIG. 8.—Potency of eluates plotted against percentage decrease in volume of original serum. Concentrated serum does not behave as an eluate.

The factor, or factors responsible for the thrombogenic properties of serum eluates are as yet unidentified and preliminary electrophoretic investigation has shown differences in their protein concentration and composition. If the factor responsible for their thrombogenic activity is a protein it may be present either alone or attached to one of the protein fractions already demonstrated, in amounts too small to be revealed by this electrophoretic method.

The use of more sensitive qualitative and quantitative techniques as well as the injection of the various protein fractions, either singly or in combination, may prove informative, and the investigation is proceeding along these lines.

The injection of volumes of unconcentrated serum, sufficient to produce thrombus, caused increases of up to 50 per cent in the blood volumes of the recipient animals (Fig. 4). The failure of whole serum to produce the pattern of thrombus formation seen with even a dilute eluate may be due either to the

marked dilution of the blood of the recipient animals leading to a possible retardation of the coagulation, or to an inhibitor present in serum but not in eluates, and the failure of concentrated serum to maintain massive and total thrombus formation in successive animals in the same way as its eluate (Fig. 6) supports the latter view.

This failure of concentrated serum to behave as an eluate is further demonstrated in Fig. 8, the lowest concentration of C units producing thrombus being plotted against the percentage decrease in volume suffered by the original sera in the production of their eluates. The concentrated serum, which had undergone a reduction in volume of 66 per cent, was less potent than eluates whose original sera had undergone similar reductions, and the similarity of results obtained with serum after freezing and freeze-drying respectively indicates that the latter process had no inhibitory action on the serum and was therefore not responsible for the lower potency of the concentrated batch.

Unconcentrated sera demonstrated a curious periodicity of thrombus formation, the peaks occurring at the same levels of C units using the same serum. This suggests that in thrombus formation using whole serum, activators and inhibitors both in the injected serum and in the blood of the recipient animal must be present in optimum proportions for thrombus formation to occur either at all or in any quantity. A similar periodicity was seen with concentrated serum suggesting that dilution of the blood of the recipient animals played little, if any, part in the phenomenon.

Two out of three batches of pooled serum first produced thrombus at approximately the same level of C units (Fig. 4 and 5A) while the third achieved this at a considerably lower concentration (Fig. 7). Although this may represent a wide variation of the normal, it is possible that one or more of the sera participating in the latter batch were unduly thrombogenic.

SUMMARY

The thrombogenic properties of human serum eluates and whole sera were determined quantitatively *in vivo* by assessing their thrombus production in the occluded portal veins of rats.

From the uniformity of the results obtained with individual eluates and sera, it would appear that differences between different eluates and sera were inherent in those materials and were not due to experimental variation.

The potency of eluates was broadly inversely related to their dilution, although an occasional sample was unduly thrombogenic, due presumably to the abnormal potency of the original serum.

Apart from convertin, the factors responsible for the thrombogenic properties of serum eluates are, as yet, unidentified and preliminary electrophoresis showed the latter to vary in protein concentration and composition. However, more sensitive qualitative and quantitative methods as well as the injection of protein fractions, singly or in various combinations, may reveal the fraction or fractions responsible.

Experimental evidence pointed to the presence of an inhibitor to coagulation present in serum but not in eluates, and suggested that activators and inhibitors must be present in optimum proportions for maximum thrombus production to occur.

It would appear, therefore, that the quantitative adaptation of the method of producing intravascular coagulation devised by Wessler (1955) can be used for the biological assay of the coagulability imparted by human serum and serum fractions and affords a method not only for investigating the coagulation mechanism from a new angle but for the possible detection of unduly thrombogenic sera, a procedure of considerable clinical value.

My thanks are due to Dr. W. J. D. Fleming for his valuable advice and criticism, and to Miss Grodzinski-Barthen for technical assistance.

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EXPERIMENTAL PYELONEPHRITIS:
THE RELATIONSHIP OF BACTERIAL VIRULENCE TO THE ESTABLISHMENT OF THE RENAL LESION

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ONE of the remarkable findings in urinary tract infections of man is that the organisms responsible for the majority of primary infections are of the *Bacterium* genus (Garrod, Shooter and Curwen 1954 ; Beeson 1955 ; Brod 1956). Infections which follow catheterization and operative procedures are associated with a wider range of bacteria but *Bacterium* species still predominate.

If coagulase positive staphylococci are injected intravenously into a normal animal they multiply to produce abscesses in the kidney (Navasquez 1950), whereas most other bacterial species are unable to do so. However, if the flow of urine from the kidney is obstructed by ureteric ligation *Bacterium coli* will localize and multiply (Lepper, 1921). This is not due to increased trapping of organisms (Guze and Beeson, 1956) and the progression of the lesion is dependent upon the maintenance of the ureteric obstruction (Brumfitt and Heptinstall, 1958).

The possibility that the bacterial species associated with pyelonephritis may possess special properties which enable them to multiply in the kidney has received little attention. In the present work different strains of *Bact. coli* have been compared for their ability to produce renal infection in the rat. The bacterial strains used have been investigated serologically, examined by conventional pathogenicity tests, tested for susceptibility to the bactericidal action of rat serum, the *in vitro* growth rate determined and the chemical components of the bacterial cell walls estimated. This characterization of the individual bacteria was carried out to discover whether any of the findings correlate with ability to produce renal lesions.

METHODS

Bacterial strains.—Four strains of *Bact. coli* were used. The first was that used in previous investigations (Brumfitt and Heptinstall, 1958, 1959) and was originally isolated from a patient with acute pyelonephritis. The antigenic structure of this strain was O77 : H13 and in the text will be referred to as C_3. The second, a strain carrying a K-antigen O9 : K28, was kindly supplied by Dr. Ørskov of the State Serum Institute, Copenhagen and was designated K28. The third strain O111 : B4 : H12 was a mouse pathogenic strain obtained from Dr. D. Rowley of the Wright-Fleming Institute. Throughout the text this strain will be referred to as CV. The fourth organism was a rough strain of *Bact. coli* (designated " Lilly ") supplied by Dr. A. C. Wardlaw of the Wright-Fleming Institute.

The laboratory strain of *Chromobacterium prodigiosum* served as a control organism.

To facilitate identification of these bacteria after animal inoculation, detailed biochemical studies were carried out and the bacteria marked genetically by making them resistant to streptomycin (100 μg. per ml.).

Test of ability to colonize kidney.—Storage of the organisms, preparation of cultures for animal inoculations and the method for counting bacteria in the kidneys have been described before (Brumfitt and Heptinstall, 1958).

Female albino Wistar rats (Glaxo) of 200 g. were anaesthetized, the left ureter exposed and occluded by means of a silk thread. After an interval of 20 min. the test organism was injected intravenously. The technique of injection of organisms into the rat was to expose the jugular vein under light ether anaesthesia, inject 0·5 ml. of bacterial suspension (approx. 200×10^6 organisms) and close the wound with a continuous suture. After 5, 15 min., 1, 2, 12, 24 hr. and 4 days groups of animals were killed by overdose of ether and their left kidneys removed and total bacterial counts carried out.

In vitro *bactericidal action of serum.*—Serum was obtained from a group of normal rats. These were anaesthetized, the thorax opened and blood withdrawn from the heart by piercing the ventricle with a sterile Pasteur pipette. The blood was allowed to stand until the clot retracted and the serum pipetted off. Tests were carried out within 24 hr. of obtaining the serum. A 1 : 3 dilution of the serum was prepared in saline buffered to pH 7 and warmed to 37°. To 2·5 ml. of the diluted serum was added approximately 3×10^4 of the test organism suspended in 0·5 ml. buffered saline and in the log. phase of growth. After mixing, 0·2 ml. was withdrawn and a viable count performed to give a zero reading. Further samples for viable counts were taken at 15 min., 30 min. and 60 min.

Pathogenicity tests.—These were carried out by intraperitoneal injection of groups of 5 mice whose individual weights were 18–20 g. Four separate tests were done with each organism and all results used in the final analysis, the LD_{50} being calculated by the method of Reed and Muench (1938). Bacterial suspensions were made in saline to which hog mucin had been added to a final concentration of 1·7 per cent. Viable counts on the bacterial suspensions were carried out in every case.

Preparation of bacterial cell walls.—Litwack and Pramer (1956) have shown that variation in the chemical composition of culture medium can influence the cell wall composition of *Micrococcus lysodeikticus*. Experiments with the *Bact. coli* strains showed that not only the composition of the medium but time of growth caused marked variation in cell wall composition. Therefore, the growth of bacteria for cell wall preparation was controlled by using the same batch of nutrient agar, using a standard inoculum and harvesting bacteria after 18 hr. incubation at 37°. The bacteria were filtered through glass-wool and washed in distilled water.

Cell walls were prepared by treatment of heavy bacterial suspensions (approximately 10^{10} organisms per ml.) in the Mullard sonic generator, care being taken to use the minimum time necessary for distintegration of the bacteria. The basic techniques used for preparation of cell walls have been described previously (Brumfitt, 1959).

Cell wall analysis.—Lipopolysaccharide was estimated by extraction of freeze-dried cell walls with 90 per cent phenol water at 65° after the method of Westphal, Lüderitz and Bister (1952). Protein was measured by the biuret method. Following acid hydrolysis, hexosamine (Rondle and Morgan, 1955) and phosphorus (Allen, 1940) were estimated.

In vitro *growth curve.*—Bacteria in the log phase were diluted with nutrient broth at 37° so as to give a final concentration of approx. 1000 organisms per ml. The culture was agitated mechanically and samples removed at 30 min. intervals. Viable counts were carried out by preparing dilutions which were plated on to nutrient agar.

RESULTS

Counts carried out after 24 hr. and 4 days on rat kidneys whose ureters had been ligated to permit bacterial proliferation are shown in Table I. It can be seen that counts for the Lilly strain were low compared with C_3, CV, K_{28} and *Chr. prodigiosum*. Similar lower counts with Lilly were obtained at 5, 15, 60, 120 min. and 12 hr. Examination at 4 days showed that 10 out of the 12 obstructed kidneys exposed to infection by Lilly contained no viable bacteria and in the 2 kidneys where the organism had multiplied no abscess formation had resulted. Strains C_3, CV and K_{28} all caused pyelonephritis although abscess formation was more extensive with the 2 former organisms. This correlated with the finding that at 4 days counts obtained for C_3 and CV were significantly higher than those for K_{28} (Table I). *Chr. prodigiosum* produced counts similar to those for CV and K_{28} after 24 hr. but the infection was not sustained and at 4 days high counts

TABLE I.—*To Show the Bacterial Content of the Left Kidneys at Intervals Following Intravenous Injection of Organisms*

Time of killing	*Bact. coli* C_3 ($\times 10^6$)	*Bact. coli* CV ($\times 10^6$)	*Bact. coli* K_{28}	*Bact. coli* Lilly	*Chr. prodigiosum*
24 hr.	11·0	28·3	$7·0 \times 10^6$	0	$3·08 \times 10^6$
	75·0	8·2	$11·32 \times 10^6$	0	$14·44 \times 10^6$
	22·0	9·2	$7·96 \times 10^6$	200	$1·909 \times 10^6$
	17·0	4·2	$2·3 \times 10^4$	400	$12·58 \times 10^6$
	62·4	0·04	$4·5 \times 10^6$	0	$6·1 \times 10^6$
	23·2	8·8	$8·18 \times 10^6$	100	$4·16 \times 10^6$
	34·0	99·0	$1·82 \times 10^6$	0	$6·84 \times 10^6$
	46·0	6·3	$1·72 \times 10^6$	0	$1·57 \times 10^6$
	19·0	6·9	$12·6 \times 10^6$	0	$7·76 \times 10^6$
	29·0	3·59	$15·6 \times 10^6$	0	$2·8 \times 10^3$
	30·0	3·29	$7·6 \times 10^6$	0	$1·6 \times 10^3$
	31·0	23·0	$7·9 \times 10^5$	$7·84 \times 10^5$	$9·0 \times 10^3$
4 days	43·2	70·4	$9·78 \times 10^5$	$6·04 \times 10^4$	20
	422·0	90·3	$28·0 \times 10^6$	0	0
	172·0	150·0	$24·0 \times 10^6$	0	0
	120·0	326·0	$14·0 \times 10^6$	0	$3·0 \times 10^5$
	88·0	0·93	$105·0 \times 10^6$	0	0
	704·0	40·5	$147·4 \times 10^6$	0	$46·6 \times 10^6$
	58·0	76·0	$35·0 \times 10^6$	0	0
	48·0	23·0	$1·1 \times 10^6$	0	200
	84·0	440·3	0	0	0
	64·0	78·2	$9·52 \times 10^6$	0	$7·06 \times 10^6$
	38·0	10·5	$4·6 \times 10^5$	$1·57 \times 10^5$	0
	148·0	320·0	180	0	$6·76 \times 10^6$

Analysis of Results

24 hr.	$C_3 > K_{28}$	$P < 0·001$
	$C_3 >$ CV	$P < 0·01$
	$C_3 >$ *Chr. prodigiosum*	$P < 0·001$
	CV + K_{28}.	No difference ($0·5 > P > 0·4$)
	CV + *Chr. prodigiosum*.	No difference ($0·1 > P > 0·05$)
	K_{28} + *Chr. prodigiosum*.	No difference ($0·3 > P > 0·2$)
4 days	$C_3 > K_{28}$	$P < 0·001$
	CV $> K_{28}$	$P < 0·01$
	C_3 + CV.	No difference ($0·4 > P > 0·3$)

were found in only 4 of the 12 kidneys and 6 of the kidneys were sterile. Abscess formation was developed in only one of the kidneys infected with *Chr. prodigiosum*. The statistical appraisal of the results is shown at the foot of Table I, the calculations being based upon the techniques advocated by Bartlett (1947).

It was found that the average generation time *in vitro* did not vary significantly for any of the *Bact. coli* strains used in the present experiment. This shows that an inherent ability to multiply more rapidly was not the reason for some *Bact. coli* strains being able to colonize the kidney more successfully than others. *Chr. prodigiosum* had a significantly longer average generation time than the *Bact. coli* strains.

Table II summarizes the results of the serum bactericidal tests. It is seen that *Bact. coli* Lilly is rapidly destroyed by the rat serum whilst *Chr. prodigiosum* succumbs only gradually. C_3, CV and K_{28} are resistant and in fact continue to multiply when suspended in rat serum.

The results of the virulence tests on mice are shown in Table III. Although the test was carried out with the addition of hog mucin, it can be seen that, apart

from CV, the strains were of low pathogenicity for mice. The hog mucin produced little augmentation in virulence of the latter strains compared with suspension in physiological saline alone.

Chemical analysis of the cell walls of the coli strains is given in Table IV. The most obvious difference between the strains is in the lipopolysaccharide content which is high in strain C_3 (18·4 per cent) and CV (16·8 per cent) and much lower in K_{28} (8·9 per cent) and Lilly (7·5 per cent).

TABLE II.—*Survival of* Bact. coli *Strains and* Chr. prodigiosum *in Rat Serum at* 37°

Interval after mixing bacteria and serum (min.)	*Bact. coli* C_3	*Bact. coli* CV	*Bact. coli* K_{28} $\times 10^3$	*Bact. coli* Lilly	*Chr. prodigiosum*
0	5·5	2·5	2·7	3·2	4·0
15	5·1	3·2	3·2	0	2·2
30	7·7	3·4	3·9	0	1·4
60	12·9	4·0	4·2	0	0·6

Figures in the table refer to the number of viable organisms per ml.

TABLE III.—LD_{50} *Values for the Various Strains of Bacteria Following Intraperitoneal Inoculation into Mice*

Strain	LD_{50}
C_8	5×10^8
CV	7×10^6
K_{28}	8×10^8
Lilly	8×10^9
Chr. prodigiosum	3×10^9

TABLE IV.—*Composition of* Bact. coli *Cell Walls*

Strain	Lipopoly-saccharide	Hexosamine	Phosphorus	Protein
C_3	18·4	1·84	1·6	63
CV	16·8	2·9	1·8	50
K_{28}	8·9	2·1	2·0	64
Lilly	7·5	1·3	1·4	66

The figures in the Table refer to percentage by weight.

DISCUSSION

Following intravenous injection of *Bact. coli* into rats or rabbits the bacteria are rapidly removed and destroyed by the reticulo-endothelial system. Even after injection of large numbers of organisms only a small proportion localize in the kidney (Gorrill, 1956). Normally *Bact. coli* strains do not multiply and are slowly destroyed; whereas if ureteric ligation has been performed, after a lag period multiplication begins in the kidney (Guze and Beeson, 1956; Brumfitt and Heptinstall, 1958). Beeson and Rowley (1959) have demonstrated that renal tissue has an anticomplementary effect greatly in excess of other organs of the body and it is possible that the inactivation of the serum bactericidal mechanisms is one of the important factors in determining survival of bacteria

which localize in the kidney. Owing to destruction by serum, *Bact. coli* Lilly was found in the kidney in much smaller numbers than C_3 or *Chr. prodigiosum* at 5, 15, 60 and 120 min. and the survival of these few organisms must have been due to interference with the serum bactericidal mechanism by the anticomplementary activity of the kidney since when suspended in rat serum Lilly was completely killed within 15 min. (Table II). From the data available it is not possible to say whether Lilly would be capable of abscess formation if greater numbers of this organism had localized initially in the kidney. However, our findings suggest that the properties of the individual bacteria as well as host resistance are important in the development of pyelonephritis. Obstructed kidneys examined macroscopically 4 days after the inoculation of organisms showed an obvious difference in severity of the infection produced by the bacterial strains. It varied from the development of severe infection in all cases with C_3 and CV to the failure of Lilly to produce any macroscopical change. These changes were reflected in total bacterial counts in the kidneys at 4 days which show a significant difference between all of the groups except C_3 and CV (Table I). This finding suggests that the bacteria in the obstructed kidney differ in their ability to resist destruction or in their ability to multiply. *Chr. prodigiosum* was able to reach high counts after 24 hr. but, in contrast to C_3, CV and K_{28}, at 4 days the kidney count had shown a significant decline (Table I). It is possible that the destruction of *Chr. prodigiosum* between 24 hr. and 4 days is caused by intracellular destruction in phagocytes which migrate into the kidney, although at present we have no proof of this. Furness (1958) found that for *Salmonella typhimurium* the virulence of a strain depended upon its ability to multiply in macrophages and eventually to kill them. This was in contrast to avirulent strains which were unable to multiply intracellularly. The difference found between *Chr. prodigiosum* and *Bact. coli* strains C_3, CV and K_{28} could be explained on a similar basis.

No clear relationship was found between mouse virulence and the ability of a bacterial strain to produce pyelonephritis in the rat. Four of the 5 bacterial strains used in the present experiment were of low virulence as judged by the mouse intraperitoneal killing test (Table IV). The mouse virulent strain (CV) was effective in the production of pyelonephritis but not superior to strain C_3 which was avirulent for mice (Table I). It may be argued with some justification that apart from variation in susceptibility of different animal species, a lack of correlation between a short term killing test and the ability to produce a lesion of the progressive type, such as pyelonephritis, is not altogether unexpected.

We are unaware of any other study in which *Bact. coli* strains have been compared for their ability to produce renal infection. Kauffman (1947, 1954) found that K-antigens were of importance in determining the toxicity of *Bact. coli* for mice and also that strains causing severe urinary tract infection in the human often possessed this antigen. In our experiments K- antigen was not of fundamental importance in the aetiology of renal infection since strain C_3 did not possess detectable K-antigen although this organism produced more severe renal lesions than strain K_{28} which did.

Because of the close relationship of lipopolysaccharide to somatic antigen and bacterial endotoxin (Westphal and Lüderitz, 1954) and the possibility that somatic antigen forms the substrate for the serum bactericidal mechanism (Rowley and Wardlaw, 1958), cell wall lipopolysaccharide was estimated quantitatively in the 4 strains of *Bact. coli* used for the experiments. It is of interest that strains C_3

and CV which contained the greatest concentration of lipopolysaccharide (Table IV) produced the most severe renal lesions and gave the highest bacterial counts. However, K_{28} which had a relatively small amount of lipopolysaccharide in the cell wall produced well developed pyelonephritis whilst Lilly, which had only slightly less, did not. Although total lipopolysaccharide content of the cell wall may have some influence on the ability of an organism to localize and multiply in the kidney with ureteric obstruction, qualitative changes in the lipopolysaccharide are probably also of importance. Davis, Staub, Fromme, Lüderitz and Westphal (1958) have shown how the specificity of the somatic antigens of the salmonellae depend upon a single sugar in the lipopolysaccharide and consequently quite small chemical changes in cell wall may profoundly alter the properties of an organism. Further investigation may reveal that similar changes in the cell wall composition of *Bact. coli* species have an important bearing upon pathogenicity.

SUMMARY

Smooth strains of *Bact. coli* (C_3, CV and K_{28}) were capable of producing progressive infection in the rat kidney with an obstructed ureter if injected intravenously, but strains C_3 and CV produced a more severe infection than K_{28}. The latter organism carried a K-antigen whereas C_3 did not and it was concluded that the presence of this antigen was not of fundamental importance in the production of pyelonephritis. Intravenous injection of a rough strain of *Bact. coli* (Lilly) did not produce a progressive infection and this was due to the great susceptibility of the organism to the non-immune serum bactericidal system. *Chr. prodigiosum* was able to multiply in the obstructed kidney and gave high counts at 24 hr. A decline then took place in the majority of cases and abscess formation rarely occurred. This was unlikely to be related to the direct action of the serum bactericidal system but destruction by phagocytosis may be the explanation.

The bacterial strains were tested for mouse virulence by intraperitoneal inoculation. These results were compared with the ability to produce progressive renal infection but no correlation was found.

Strains C_3 and CV which produced the most severe kidney lesions contained considerably more cell wall lipopolysaccharide than the other *Bact. coli* strains but there is not a simple relationship between cell wall lipopolysaccharide content and ability to produce infection.

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SURVEY OF PAPERS

NISBET, BARBARA HESLOP and IRMGARD ZEISS induced immunochemical tolerance in Wistar rats by the subcutaneous injection of spleen cells from hooded donors within 24 hr. of birth. When the recipients became adult they were grafted with standard implants of cortical bone from the original donors. These implants and adjacent host tissues were examined histologically at varying times from 2–250 days.

A high degree of tolerance was induced in 36·5 per cent and a moderate tolerance in 28 per cent of the recipients, while 21 per cent of the animals died prematurely. In the tolerant animals the reaction of the host to the homografts was modified in that it approximated to the usual reaction seen around autografts and the type of reaction could be correlated with the degree of conferred tolerance in the animals (p. 443).

SMITH, KEPPIE, COCKING and WITT have obtained from guinea-pigs dying from plague up to 6·8 g. of *Past. pestis* in sufficient purity to study the chemical basis of virulence and immunogenicity. *In vivo* products of the organisms enhanced the virulence of *Past. pestis* for guinea-pigs, and inhibited phagocytosis by guinea-pig and mouse leucocytes. Virulent organisms produced 2 precipitinogens, X and Y, in addition to V and W *in vivo* and in some *in vitro* conditions (p. 452).

COCKING, KEPPIE, WITT and SMITH, continuing their work on the virulence of *Past. pestis*, show that products prepared from numbers of the organisms less than the numbers found in guinea-pigs which died of plague are fatal to healthy animals. Material was obtained by ultrasonic treatment and chemical purification. They found that material toxic for mice was predominantly in the soluble extract while that toxic for guinea-pigs was unequally divided between the soluble extract and the residue (p. 460).

BYERS, KING and HARRISON produced experimental infective pneumoconiosis in Hartley strain guinea-pigs with vole bacillus mixed with coal or kaolin dust. They found that animals treated with Triton developed the same lesions as controls, but that this substance prevented necrosis and eliminated bacteria from the lesions. Interpretation of their results was complicated by an outbreak of pseudotuberculosis in their animals (p. 472).

OHSAKA, IKEZAWA, KONDO, KONDO and UCHIDA fractionated the venom of the crotalid snake Habu (*Trimeresurus flavoviridis*) electrophoretically in a starch column. They examined fractions of eluate from the column for protein, proteolysis of casein, haemorrhage induction in rabbit skin and lethal toxicity for mice. From their results they endeavour to distinguish between factors responsible for the venom's various effects (p. 478).

WILHELM and BRENDA MASON show that increased permeability induced by minimal thermal stimuli was biphasic in guinea-pigs, rats and rabbits. There was an immediate response maximal in 5 min., followed by delayed response maximal at 3–4 hr. In the guinea-pig and rabbit the immediate response was mediated by histamine. The immediate response in the rat was only moderately suppressed by antihistamines. The delayed response in all species was unaffected by antihistamines. The possible role of globulin permeability factor as mediator of the delayed component could not be confirmed, because, possibly through technical difficulties, the response was not inhibited by potent *in vivo* antagonists of globulin PF (p. 487).

TAMAR GOTLIEB-STEMATSKY and SOPHIE LEVENTON grew polyoma virus in monolayers of cells prepared from whole mouse embryos. They obtained plaques of different sizes and isolated strains of virus from the small and large plaques. The former was of marked cytopathogenicity, but slight haemagglutinating and oncogenic capacity, and was named C^+H^-; the latter was less cytopathogenic, but actively haemagglutinating and tumour inducing and therefore called C^-H^+. The two strains did not differ antigenically (p. 507).

NORTH, SANDERS and FLOREY transplanted lengths of duodenum into the subcutaneous tissues of the abdomens of rabbits in an investigation into the blood supply of the trans-

planted tissues. It was found that adequate vascular connections, both arterial and venous, were formed between the vessels and the transplanted duodenum and those of the surrounding subcutaneous tissues. In most of the cases, when the pedicle of the transplant was subsequently divided, the duodenum remained viable and continued to secrete typical rabbit duodenal juice (p. 520).

REESE examined the lower edge of the left lateral lobe of the liver in anaesthetized Wistar rats by incident ultraviolet light, using a $\times 22$ water immersion objective. During the observations sodium fluorescein was introduced into the animal's circulation through a cannula in the jugular vain. The time taken by this substance to pass from sinusoids to liver cells and thence to bile canaliculi was noted. Similar data were recorded for hypoxic rats which had breathed nitrogen-oxygen mixtures containing 6–12 per cent of oxygen for 1 hr. before receiving fluorescein. It was found that hypoxia slowed the passage of fluorescein into the bile canaliculi and caused other changes which the author describes (p. 527).

JULIA FOURMAN investigated the effects of injecting chondroitin sulphate in nephrocalcinosis of the rat. Nephrocalcinosis produced by parathyroid extract is associated with the deposition of a material in the kidney having the histochemical characteristics of a polysaccharide.

The author found that CS injected into rats produced casts in the collecting tubules and an increase of Alcian blue staining material in the glomeruli and in the medullary interstitial tissues. Similar changes were produced by the injection of small doses of parathyroid extract. However, the injections of CS did not affect the calcium content of the kidney nor did they modify the changes induced by the injection of calcium gluconate (p. 536).

SCOTT produced thrombosis of the portal vein of male Wistar rats by injection of human serum or eluates of barium sulphate absorbed convertin-rich fractions. A bio-assay method for determining quantitatively the thrombogenic properties of these is described. Some evidence of a coagulation inhibitor was found in whole serum but not in the eluates prepared from the same serum (p. 543).

BRUMFITT and HEPTINSTALL, continuing their work on experimental pyelonephritis, have shown that a smooth strain of *Bact. coli* carrying the K antigen gave a less severe infection than strains in which it was absent. Further they showed that a rough strain of *Bact. coli* did not produce progressive infection. *Chr. prodigiosum* was able to multiply in the obstructed kidney, but abscess formation rarely occurred (p. 552).